

REMARKS

Enclosed herewith in full compliance with 37 C.F.R. §§1.821-1.825 is a Second Substitute Sequence Listing to be inserted into the specification as indicated above. The Second Substitute Sequence Listing in no way introduces new matter into the specification. Also submitted herewith in full compliance with 37 C.F.R. §§1.821-1.825 is a disk copy of the Second Substitute Sequence Listing. The disk copy of the Second Sequence Listing, file "2003-06-09 1110-0266P.st25.txt", is identical to the paper copy, except that it lacks formatting. No new matter is introduced by these amendments. The specification has been amended to insert SEQ ID NO:17 into the Second Substitute Sequence Listing. Support for SEQ ID NO:17 is discussed below.

Rejections under 35 U.S.C. §112, first paragraph

The Examiner maintains the rejection of claims 2, 3, 6, 10, 11 and 12 under 35 U.S.C. §112, 1<sup>st</sup> paragraph, for lack of written description. It appears to Applicants that the Examiner maintains the rejection on the basis of a lack of a reference sequence for the natural human Fas ligand. The Examiner is of the position that the sequence for "natural human Fas ligand" is essential to the invention; therefore must be designated with a specific sequence. The Examiner further notes that the Applicants have not conclusively shown that there is only one known naturally occurring human Fas ligand.

Applicants traverse this rejection and withdrawal thereof is

respectfully requested.

The present invention, as encompassed by claim 2 is drawn to an isolated polypeptide

- 1) having an amino acid sequence of natural human Fas ligand
- 2) wherein the 129<sup>th</sup> amino acid and 130<sup>th</sup> amino acid residues as measured from N terminal end are both deleted,
- 3) and at least one amino acid residue from 111<sup>th</sup> amino acid to 128<sup>th</sup> amino acid residues or at least one amino acid residue from 131<sup>st</sup> amino acid to 133<sup>rd</sup> amino acid residues as measured from N terminal end is deleted.

Independent claims 3, 10 and 11 also recite various features defining the derivative of human Fas ligand polypeptide of the invention. The full Fas ligand sequence was known prior to the present invention. Thus, by recitation of "natural human Fas ligand" one skilled in the art would have been well aware of the sequence being recited.

One skilled in the art would have readily recognized that at the time of the invention that the recitation of "natural human Fas ligand" in the specification referred to a specific amino acid sequence. The specification references at least on pages 1-3 various journal articles that discuss the human Fas ligand. The Sequence Listing has been amended to add the sequence of the human Fas Ligand to the Sequence Listing as SEQ ID NO:17. In addition, the claims have been amended to reference SEQ ID NO:17.

The addition of SEQ ID NO:17 to the specification and claims

is not new matter because the originally filed claims and specification reference "natural human Fas ligand." It was well-recognized in 1997 (the time of the invention) that natural human Fas ligand protein was the protein of Accession No. AAC50124. Therefore, the sequence is inherent in the reference to "natural human Fas ligand." Evidence that one skilled in the art would have identified SEQ ID NO:17 as the sequence of human Fas ligand in 1997 is attached hereto as Exhibits (1)-(5), the significance of each is discussed below.

(1) "OMIM" (Online Mendelian Inheritance in Man): This reference details the history of the cloning of the human Fas ligand in 1994. Page 2, 2<sup>nd</sup> paragraph, first sentence of the OMIM reference indicates that the amino sequence of human Fas ligand is disclosed in Takahashi et al. *International Immunology*, Vol. 6, No. 10, pp. 1567-1574 (1994), which is attached as Exhibit (2).

(2) Takahashi et al. *International Immunology*, Vol. 6, No. 10, pp. 1567-1574 (1994):

Figure 1 of Takahashi et al. discloses the amino acid sequence of human Fas ligand, which is the sequence of SEQ ID NO:17. The amino acid sequence of Figure 1 of Takahashi et al. is also registered on the NCBI (Exhibit 3). Takahashi et al. is cited on page 42, line 2 of the present specification.

(3) NCBI listing: Exhibit 3 is the NCBI listing of human Fas ligand, which is also the sequence of SEQ ID NO:17.

(4) WO 95/18819: The WO '819 publication is an International patent application was filed by a different set of inventors than the authors of Exhibit (2), Takahashi et al. WO '819 demonstrates that multiple investigative groups cloned natural human Fas ligand between 1994 and 1995. Sequence ID No. 2 of WO '819, human Fas ligand, is the same sequence disclosed in the other sources and listed in the present application as SEQ ID NO:17.

(5) Nagata et al. Science Vol. 267, pp 1449-1456 (1995): Nagata et al. is a review article that published in 1995 and discussed Fas and Fas ligand. In Nagata et al., Exhibit (2) above, Takahashi et al., is cited for the cloning of natural human Fas ligand. See page 1450, right column 8<sup>th</sup> from final line; page 1451, left column line 16; and page 1455, right column, 3<sup>rd</sup> line. The citation to Takahashi et al. evidences that one skilled in the art recognized natural human Fas ligand as being the protein disclosed in Takahashi et al., i.e. the protein having the amino acid sequence of SEQ ID NO:17.

These references clearly demonstrate that at the time of the invention, natural human Fas ligand was recognized by those skilled in the art as being the protein of SEQ ID NO:17. As such, addition of this sequence to the Sequence Listing and reference

of this SEQ ID NO: in the claims is fully supported by the originally filed disclosure and is not new matter. This amendment further addresses the rejection for lack of written description and withdrawal of the rejection is respectfully requested.

**Rejections under 35 U.S.C. 112, second paragraph**

Claims 8 and 9 have been rejected under 35 U.S.C. §112, second paragraph with the indication that the term "novel" must be deleted from the claims and replaced with "isolated" or "purified." Claims 5, 8 and 9 have been amended as requested by the Examiner to recite "An isolated DNA...." As such, withdrawal of the rejection is respectfully requested.

As the above-indicated amendments and remarks address and overcome the objections and rejections of the specification and claims, withdrawal of the objections and rejections and allowance of the claims are respectfully requested.

Should the Examiner have any questions regarding the above-indicated application she is requested to please contact MaryAnne Armstrong, PhD (Reg. No. 40,069) in the Washington DC area at (703) 205-8000.

A marked-up version of the amended claims showing all changes is attached hereto.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional

Docket No. 1110-266P

fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17;  
particularly, extension of time fees.

Respectfully submitted,

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Attachments: Version with Markings to Show Changes Made  
Exhibits (1)-(5)

VERSION WITH MARKINGS TO SHOW CHANGES MADE  
IN THE SEQUENCE LISTING

The sequence listing has been amended to add new SEQ ID NO:17.

IN THE CLAIMS

Claims 2, 3, 5, 6, and 8-12 have been amended as follows.

2. (Four times amended) An isolated polypeptide having an amino acid sequence of natural human Fas ligand (SEQ ID NO:17) wherein the 129<sup>th</sup> amino acid and 130<sup>th</sup> amino acid residues as measured from N terminal end are both deleted, and at least one amino acid residue from 111<sup>th</sup> amino acid to 128<sup>th</sup> amino acid residues or at least one amino acid residue from 131<sup>st</sup> amino acid to 133<sup>rd</sup> amino acid residues as measured from N terminal end is deleted.

3. (Four times Amended) An isolated polypeptide having an amino acid sequence of natural human Fas ligand (SEQ ID NO:17) wherein all of the 8<sup>th</sup> amino acid to 69<sup>th</sup> amino acid residues as measured from N terminal end are deleted, 129<sup>th</sup> amino acid and 130<sup>th</sup> amino acid residues as measured from N terminal end are both deleted, and at least one amino acid residue from 111<sup>th</sup> amino acid to 128<sup>th</sup> amino acid residues or at least one amino acid residues from 131<sup>st</sup> amino acid to 133<sup>rd</sup> amino acid residues as measured from N terminal end is deleted.

5. (Twice Amended) A An isolated DNA coding for the novel polypeptide of claim 2.

6. (Thrice Amended) A soluble Fas ligand which inhibits Fas-mediated apoptosis and which comprises the amino acid sequence represented from Gln of the 130<sup>th</sup> amino acid to C terminal amino acid residue as measured from N-terminal end of natural human Fas ligand (SEQ ID NO:17).

8. (Amended) A An isolated DNA coding for the novel polypeptide of claim 3.

9. (Amended) A An isolated DNA coding for the novel polypeptide of claim 4.

10. (Four times amended) An isolated polypeptide having an amino acid sequence of natural human Fas ligand (SEQ ID NO:17) wherein the 129<sup>th</sup> amino acid and 130<sup>th</sup> amino acid residues as measured from N terminal end are both deleted, and at least one amino acid residue from 111<sup>th</sup> amino acid to 128<sup>th</sup> amino acid residues or at least one amino acid residue from 131<sup>st</sup> amino acid to 133<sup>rd</sup> amino acid residues as measured from N terminal end is deleted, wherein said polypeptide has membrane binding activity and induces Fas-mediated apoptotic activity.

11. (Four times amended) An isolated polypeptide having an amino acid sequence of natural human Fas ligand (SEQ ID NO:17) wherein all of the 8<sup>th</sup> amino acid to 69<sup>th</sup> amino acid residues as measured from N terminal end are deleted, 129<sup>th</sup> amino acid and 130<sup>th</sup> amino acid residues as measured from N terminal end are both deleted, and at least one amino acid residue from 111<sup>th</sup> amino acid to 128<sup>th</sup> amino acid residues or at least one amino acid residues from 131<sup>st</sup> amino acid to 133<sup>rd</sup> amino acid residues as measured from N terminal end is deleted, wherein said polypeptide has membrane binding activity and induces Fas-mediated apoptotic activity.

12. (Amended) An isolated peptide having an amino acid sequence of natural human Fas ligand (SEQ ID NO:17) wherein at least four amino acid residues, including 128<sup>th</sup> and 131<sup>st</sup> amino acid residues are continuously deleted from the 111<sup>th</sup> amino acid to the 133<sup>rd</sup> amino acid residues as measured from N terminal end.

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# OMIM

Online Mendelian Inheritance in Man



Johns  
Hopkins  
University

Exhibit 1

(1)

PubMed

Nucleotide

Protein

Genome

Structure

PMC

Taxonomy

OMIM

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for

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## TUMOR NECROSIS FACTOR LIGAND SUPERFAMILY, MEMBER 6; TNFSF6

### Alternative titles; symbols

**FAS LIGAND; FASL**

APOPTOSIS ANTIGEN LIGAND 1; APT1LG1

APOPTOSIS ANTIGEN LIGAND

CD95 LIGAND; CD95L

CD178 ANTIGEN; CD178

Gene map locus 1q23

### TEXT

Life requires death. Elimination of unwanted cells is vital for embryogenesis, metamorphosis and tissue turnover, as well as for the development and function of the immune system. Mammalian development is tightly regulated not only by the proliferation and differentiation of cells but also by cell death. The cell death that occurs during development or tissue turnover is called programmed cell death, most of which proceeds via apoptosis. Apoptosis is morphologically distinguished from necrosis, which occurs during the accidental cell death caused by physical or chemical agents. During apoptosis, the cytoplasm of the affected cells condenses, and the nucleus also condenses and becomes fragmented. At the final stage of apoptosis, the cells themselves are fragmented (apoptotic bodies) and are phagocytosed by neighboring macrophages and granulocytes. Apoptosis occurs not only during programmed cell death, but also during the death process induced by some cytotoxic T cells. Suda et al. (1993) identified the ligand that triggers cell death by binding to the cell surface receptor variously known as FAS or APT1 (134637). This cell surface receptor was discovered in 1989 with the isolation of 2 monoclonal antibodies (anti-Fas and anti-Apo-1) that had the startling property of killing a human cell line used as the immunogen. Cell death occurred by apoptosis. Cloning of the genes revealed that the antigens recognized by the 2 monoclonal antibodies were one and the same. It is a transmembrane protein related to a family of receptors that includes the 2 tumor necrosis factor (TNF) receptors (191190, 191191). In mice, mutations at the lpr (lymphoproliferation) locus have a defect in the FAS antigen. The inability of homozygous mutant mice to mediate FAS-induced apoptosis provokes a complex immunologic disorder featuring defects in both the B and T lymphoid compartments. A very similar phenotype of mice homozygous for the gld (generalized lymphoproliferative disease) mutation suggested that the gld gene encodes the ligand for FAS. Suda et al. (1993) isolated the ligand from a cytotoxic T hybridoma by a sensitive expression cloning strategy. The amino acid sequence indicated that FAS ligand is a type II transmembrane protein that belongs to the tumor necrosis factor family. Northern hybridization revealed that the ligand is expressed in activated splenocytes and thymocytes, consistent with its involvement in T-cell-mediated cytotoxicity, and in several nonlymphoid tissues, such as testis. The FAS antigen is expressed not only in the cells of the immune system but also in the liver, lung, ovary, and heart, where its function is unclear.

Takahashi et al. (1994) isolated the murine Fasl gene and, by interspecific backcross analysis, localized it to the same region of mouse chromosome 1 as that occupied by the gld gene for 'generalized lymphoproliferative disease.' They showed that activated splenocytes from gld mice express Fasl mRNA. However, the Fas ligand protein in gld mice carried a point mutation in the C-terminal region, which is highly conserved among members of the TNF family. Recombinant gld Fas ligand expressed in COS cells could not induce apoptosis in cells expressing Fas. ☺



Takahashi et al. (1994) isolated the chromosomal gene for human FasL. The human gene consists of approximately 8 kb and is split into 4 exons. The human FASL cDNA predicted a type II membrane protein consisting of 281 amino acids and a calculated M(r) of 31,759 that showed 76.9% amino acid sequence identity with the mouse protein. When expressed in COS cells, both human and mouse recombinant FasL induced apoptosis, indicating crossreactivity. A sequence of approximately 300 bp upstream of the ATG initiation codon was found to be highly conserved between mouse and human. Several transcription cis-regulatory elements such as SP-1, NF-kappa-B, and IRF-1 were recognized in this region. Takahashi et al. (1994) mapped the gene to 1q23 by fluorescence in situ hybridization. ☺

Using GST pull-down analysis, Ghadimi et al. (2002) showed that the C-terminal SH3 domains of GRB2 (108355), FBP17 (606191), and PACSIN2 (604960), as well as other related proteins, bind to the polyproline-rich region of the cytoplasmic tail of FASL.

The pathogenesis of systemic lupus erythematosus (SLE; 152700) is multifactorial and polygenic. The apoptosis genes FAS and FASL are candidate contributory genes in SLE, as mutations of these genes result in autoimmunity in several murine models of SLE. In humans, FAS mutations result in autoimmune lymphoproliferative syndrome, or ALPS (e.g., 134637.0001). Wu et al. (1996) screened DNA from 75 patients with SLE by SSCP analysis for potential mutations of the extracellular domain of FASL. A heterozygous SSCP anomaly for FASL was identified in 1 SLE patient who exhibited lymphadenopathy. Molecular cloning and sequencing indicated that the genomic DNA of this patient contained an 84-bp deletion within exon 4 of the FASL gene, resulting in a predicted 28-amino acid in-frame deletion (134638.0001). A study of peripheral blood mononuclear cells from this patient revealed decreased FASL activity, decreased activation-induced cell death, and increased T-cell proliferation after activation. Lenardo (1999) expressed the opinion that although this patient satisfied the rheumatologic criteria for a diagnosis of SLE, the features were more consistent with ALPS. This might be referred to as ALPS2 or ALPS1B, the form caused by mutations in the FAS gene being designated ALPS1A. ☺

Viard et al. (1998) detected high levels of soluble FASL in the sera of patients with toxic epidermal necrolysis (TEN). Keratinocytes of TEN patients produced FASL, which induced keratinic apoptosis. Incubating keratinocytes with intravenous immunoglobulin (IVIG) completely inhibited FAS-mediated keratinocyte apoptosis. A naturally occurring anti-FAS immunoglobulin present in IVIG blocks the FAS receptor and mediates this response. Ten patients with TEN were treated with IVIG. Progression of skin disease was rapidly reversed in all cases. ☺

Hahne et al. (1996) stated that, despite the existence of melanoma-specific cytolytic T cells in tumor-infiltrating lymphocytes and in peripheral blood from melanoma patients, and the definition of 12 CTL-defined melanoma peptide antigens, melanoma cells are able to avoid immune detection in most instances. The investigators proposed that FASL-expressing melanoma cells may kill FAS-sensitive activating T lymphocytes. They analyzed FASL expression in melanoma cells and demonstrated substantial quantities of FASL in lysates of a series of human melanoma cells. Two molecular species were identified: a 40-kD membrane-bound FASL and a 27-kD extracellular FASL. Hahne et al. (1996) also demonstrated that the majority of cells infiltrating the tumors were FAS-positive. No FASL was found in normal melanocytes of the skin, suggesting that FASL upregulation occurs during tumorigenesis. Hahne et al. (1996) proposed that FASL-expressing melanoma cells might induce apoptosis of FAS-sensitive tumor infiltrating cells. They reported that injection of FasL+ mouse melanoma cells in mice led to rapid tumor formation. When FasL+ mouse melanoma cells w r injected

into FAS-deficient mutant mice, tumorigenesis was delayed. These findings led Hahne et al. (1996) to conclude that FASL may contribute to the immune privilege of tumors. They proposed further that pharmacologic products that render infiltrating T cells insensitive to FASL-induced killing may break the immunologic unresponsiveness to melanoma and provide a complementary approach in the therapy of malignant melanoma. ☺

DNA-damaged cells can either repair the DNA or be eliminated through a homeostatic control mechanism mediated by p53 (191170) termed 'cellular proofreading.' Elimination of DNA-damaged cells after UV radiation through sunburn cell (or apoptotic keratinocyte) formation is thought to be pivotal for the removal of precancerous skin cells. Hill et al. (1999) demonstrated that sunburn cell formation is dependent upon FasL. Chronic exposure to UV radiation caused 14 of 20, or 70%, of FasL-deficient mice and 1 of 20, or 5%, of wildtype mice to accumulate p53 mutations in the epidermis. Hill et al. (1999) concluded that FASL-mediated apoptosis is important for skin homeostasis, suggesting that the dysregulation of FAS-FASL interactions may be central to the development of skin cancer. ☺

In the United States more than 43,000 corneal transplants are performed each year, making it the most common form of solid tissue transplantation, and second only to bone marrow transplants in overall numbers performed. Corneal transplantation is also one of the most successful types of transplantation with failure rates at only 10 to 15% after 1 year and approximately 30% after 5 years. Stuart et al. (1997) demonstrated that the very high percentage of successful corneal transplants, without tissue matching or immunosuppressant therapy, is related to the expression of abundant functional FASL in the cornea, capable of killing FASL(+) lymphoid cells. Using a mouse model for corneal allograft transplantation, FasL (+) orthografts were accepted at a rate of 45%, whereas FasL(-) or normal grafts transplanted to Fas(-) mice were rejected 100% of the time. ☺

Pestano et al. (1999) identified a differentiative pathway taken by CD8 cells bearing receptors that cannot engage class I MHC (see 142800) self-peptide molecules because of incorrect thymic selection, defects in peripheral MHC class I expression, or antigen presentation. In any of these cases, failed CD8 T-cell receptor coengagement results in downregulation of genes that account for specialized cytolytic T-lymphocyte function and resistance to cell death (CD8-alpha/beta, see 186730; granzyme B, 123910; and LKLF, 602016), and upregulation of Fas and FasL death genes. Thus, MHC engagement is required to inhibit expression and delivery of a death program rather than to supply a putative trophic factor for T cell survival. Pestano et al. (1999) hypothesized that defects in delivery of the death signal to these cells underlie the explosive growth and accumulation of double-negative T cells in animals bearing Fas and FasL mutations, in patients that carry inherited mutations of these genes, and in about 25% of systemic lupus erythematosus patients that display the cellular signature of defects in this mechanism of quality control of CD8 cells. ☺

Grassme et al. (2000) showed that *Pseudomonas aeruginosa* infection induces apoptosis of lung epithelial cells by activation of the endogenous CD95/CD95L system. Deficiency of CD95 or CD95L on epithelial cells prevented apoptosis of lung epithelial cells *in vivo* as well as *in vitro*. The importance of CD95/CD95L-mediated lung epithelial cell apoptosis was demonstrated by the rapid development of sepsis in mice deficient in either CD95 or CD95L, but not in normal mice, after *P. aeruginosa* infection. ☺

Testis is a remarkably immune-privileged site, long known for its ability to support allogeneic and xenogeneic tissue transplants. Bellgrau et al. (1995) reported results suggesting that expression of FasL by Sertoli cells accounts for the immune-privileged nature of testis. Testis grafts derived from mice that can express functional FasL survived indefinitely when transplanted under the kidney capsule of allogeneic mice, whereas testis graft derived from mutant gld mice, which express nonfunctional ligand, were rejected. The authors speculated that FasL expression in the testis probably acts by inducing apoptotic cell death of Fas-expressing, recipient T cells activated in response to graft antigens. D'Alessio et al. (2001) demonstrated that the attribution of testicular expression of FasL to Sertoli cells

is erroneous and that FasL transcription instead occurs in meiotic and postmeiotic germ cells, whereas the protein is only displayed on mature spermatozoa. These findings point to a significant role of the Fas system in the biology of mammalian reproduction. ☺

Cytomegalovirus (CMV) is a persistent viral pathogen that resides in monocyte/macrophages and dendritic cells (DCs), critical antigen-presenting cells in the immune system. In fetal and compromised immune systems, CMV can be fatal. Raftery et al. (2001) found that recent CMV isolates, but not fibroblast-adapted CMV strains, could infect mature DCs with no change in some cell surface markers. On the other hand, flow cytometric analysis indicated a slight upregulation of the costimulatory molecules CD40 (TNFRSF5; 109535), CD80 (112203), and CD86 (601020), as well as a downregulation of MHC class I and class II molecules. Functional analysis showed that CMV-infected mature DCs suppress T-cell proliferation. Further FACS analysis demonstrated an upregulation of TRAIL (603598) and FASL, molecules that induce T-cell apoptosis through caspase (see CASP8; 601763)-dependent mechanisms, on DCs. Raftery et al. (2001) concluded that CMV evades the immune response by first downregulating MHC antigens, thereby diminishing T-cell responses, followed by an upregulation of apoptosis-inducing ligands that delete activated T cells. They also proposed that nondeletional, possibly cytokine-mediated mechanisms are involved in T-cell suppression. ☺

Mice instilled with silica develop severe pulmonary inflammation with local production of TNFA and interstitial neutrophil and macrophage infiltration in the lungs, a phenotype that resembles silicosis, an industrial era disease that afflicts certain mining professions. Borges et al. (2001) found that Fasl-deficient gld mice had reduced neutrophil extravasation into the bronchoalveolar space, did not show TNFA production increases, and did not have pulmonary inflammation in response to silica. Silica induced deferoxamine-inhibitable Fasl expression in wildtype lung macrophages *in vivo* and *in vitro*, as well as apoptosis of pulmonary macrophages. Analysis of bone marrow chimeras and local adoptive transfer experiments demonstrated that wildtype but not Fasl-deficient lung macrophages recruited neutrophils and initiated silicosis. The induction of silicosis could be blocked by the administration of neutralizing anti-Fasl antibodies. Borges et al. (2001) proposed that apoptotic cell death is required for neutrophil extravasation and pulmonary inflammation. ☺

Natural inhibitors of angiogenesis are able to block pathologic neovascularization without harming the preexisting vasculature. Volpert et al. (2002) demonstrated that 2 such inhibitors, thrombospondin I (188060) and pigment epithelium-derived factor (172860), derive specificity for remodeling vessels from their dependence on Fas/FasL-mediated apoptosis to block angiogenesis. Both inhibitors upregulated FasL on endothelial cells. Expression of the essential partner of FasL, Fas receptor, was low on quiescent endothelial cells and vessels but greatly enhanced by inducers of angiogenesis, thereby specifically sensitizing the stimulated cells to apoptosis by inhibitor-generated FasL. The antiangiogenic activity of thrombospondin I and pigment epithelium-derived factor both *in vitro* and *in vivo* was dependent on this dual induction of Fas and FasL and the resulting apoptosis. Volpert et al. (2002) concluded that this example of cooperation between pro- and antiangiogenic factors in the inhibition of angiogenesis provides one explanation for the ability of inhibitors to select remodeling capillaries for destruction. ☺

## ALLELIC VARIANTS

(selected examples)

**.0001 SYSTEMIC LUPUS ERYTHEMATOSUS, SUSCEPTIBILITY TO [TNFSF6, 84-BP DEL, EX4]**

AUTOIMMUNE LYMPHOPROLIFERATIVE SYNDROME TYPE IB

In a patient with SLE (152700) who exhibited lymphadenopathy, Wu et al. (1996) identified an 84-bp

deletion within exon 4 of the FASL gene, resulting in a predicted 28-amino acid in-frame deletion.

As stated earlier, Lenardo (1999) suggested that this patient should be classified as an instance of autoimmune lymphoproliferative syndrome (601859) due to mutation in the FASL gene. This form of ALPS has been designated ALPS1B, the form due to mutation in the FAS gene being ALPS1A.

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## Exhibit 2

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# Human Fas ligand: gene structure, chromosomal location and species specificity

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**Key words:** apoptosis, cytotoxicity, human Fas ligand gene, transcription elements

## Abstract

Fas ligand (FasL) is a 40 kDa type II membrane protein belonging to the tumor necrosis factor family, which induces apoptosis by binding to its receptor, Fas. In this report, we isolated the chromosomal gene for human FasL. The human FasL gene consists of ~8.0 kb and is split into four exons. The human FasL gene was mapped on chromosome 1q23 by *in situ* hybridization against human metaphase chromosomes. Human FasL cDNA was isolated by the reverse polymerase chain reaction of mRNA prepared from human activated peripheral blood lymphocytes.  Human FasL is a type II membrane protein consisting of 281 amino acids with a calculated M<sub>r</sub> of 31,759. It has an identity of 76.9% at the amino acid sequence level with mouse FasL. Both human and mouse recombinant FasL expressed in COS induced apoptosis in the cells expressing either human Fas or mouse Fas, indicating that FasL fully cross-reacts between human and mouse. A comparison of human and mouse FasL chromosomal genes indicated that a ~300 bp sequence upstream of the ATG initiation codon is highly conserved between them. Several transcription *cis*-regulatory elements such as SP-1, NF- $\kappa$ B and IRF-1 were recognized in this region.

## Introduction

Hormeostasis in animals is controlled not only by the proliferation and differentiation of cells, but also by cell death (1,2). Cell death during tissue development is programmed and occurs by apoptosis (3). For example, >95% of precursor T cells die by apoptosis during thymic development (4). Blood cells such as activated lymphocytes and macrophages or senescent cells in various tissues are eliminated by apoptosis after they have served their functions (1). In addition to the cell death that occurs during development, other types of cell death are also mediated by apoptosis. For example, in some cases, the cytotoxicity of cytotoxic T lymphocytes (CTL) or natural killer cells is mediated by apoptosis (5). Tumor necrosis factor (TNF) or lymphotoxin (LT) causes apoptosis of target cells (6).

Fas antigen (Fas) is a cell-surface protein belonging to the TNF/nerve growth factor (NGF) receptor family and it mediates apoptosis (7,8). We identified rat and mouse Fas ligand (FasL), and showed that FasL is a member of the TNF family (9-11). Recombinant FasL expressed in COS cells induced apoptosis by binding to Fas, indicating that FasL is a death

factor and that Fas is its receptor (12). Loss-of-function mutations of mouse Fas and FasL were identified as the spontaneous mouse mutations, *lpr* (lymphoproliferation) and *gld* (generalized lymphoproliferative disease) respectively (11,13). Since mice homozygous at the *lpr* or *gld* locus develop lymphadenopathy, and suffer autoimmune disease (14), it is likely that Fas and FasL play an important role in development of T cells.

In addition to lymphocytes, Fas is expressed in various non-lymphoid tissues such as the liver, ovary and lung (8). Fas is also expressed in various carcinoma cells (7,15). On the other hand, FasL mRNA was detected in activated splenocytes (9), and some CTL cell lines such as PC60-d10S express FasL on the cell surface and kill target cells in a Fas-dependent manner (16,17). The CTL in peritoneal exudate lymphocytes or in mixed lymphocyte culture also exert Fas-dependent cytotoxicity (16,18). These results indicate that FasL is involved in CTL-mediated cytotoxicity. Since the administration of agonistic anti-Fas antibody into mice caused hepatic failure, and rapidly killed the animals, we postulated

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that pathological tissue damage such as human fulminant hepatitis may be caused by FasL expressed in activated CTL (12,19).

In this study, we cloned the chromosomal gene and the cDNA for human FasL. The structure of the gene was similar to that of other members of the TNF family such as TNF- $\alpha$  and LT- $\beta$ . However, unlike these, which are closely arranged on human chromosome 6, the human FasL gene was localized to chromosome 1. The amino acid sequence of human and mouse FasL was well conserved, and no species-specificity was observed between human and mouse FasL.

## Methods

## Cloning of the human chromosomal gene encoding FasL

A human gene library in  $\lambda$ EMBL3 SP6/T7 constructed with DNA from human placenta was purchased from Clontech Laboratories (Palo Alto, CA). The library was screened by plaque hybridization using the  $^{32}$ P-labeled 440 bp or the 190 bp DNA fragment carrying the C-terminal (nucleotide 525-967) or N-terminal portion of rat FasL (nucleotide 43-233) respectively (9) as a probe. Hybridization proceeded under low stringency as described (11) and positive clones were plaque-purified. Recombinant  $\lambda$ DNAs were prepared as described (20), and subjected to restriction enzyme mapping and Southern hybridization analysis. Appropriate DNA fragments of the recombinant  $\lambda$ DNAs were subcloned into pBluescript II (Stratagene, La Jolla, CA), and exons were localized by Southern hybridization using rat FasL cDNA. The DNA sequence was determined using a DNA sequencer (model 370A; Applied Biosystems, Foster City, CA) and the Taq DyeDeoxy cycle sequencing kit from Applied Biosystems. Where appropriate, synthetic oligonucleotides were used as specific primers.

## Cloning of human FasL cDNA by polymerase chain reaction (PCR)

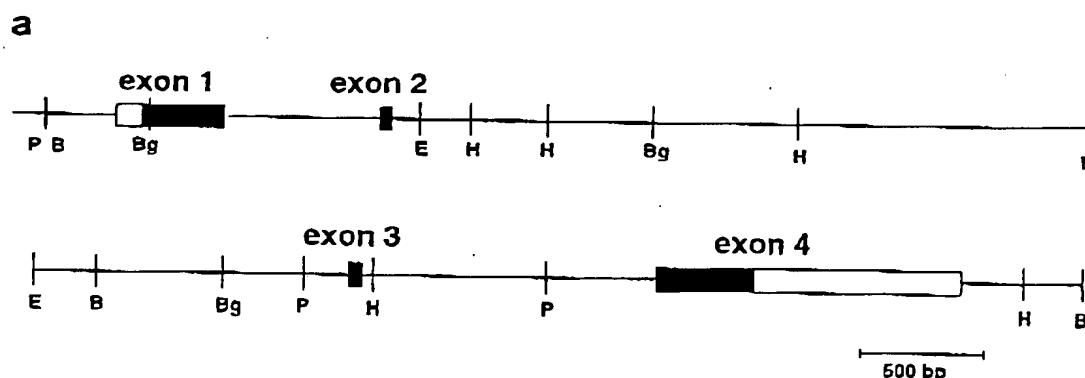
Human peripheral blood was obtained from a healthy adult volunteer (T. S.). The peripheral blood lymphocytes (PBL) were obtained by centrifugation on Nycomed solution (Nyco Prep™ 1.077; Nycomed, Oslo, Norway), and cultured in AIM-V medium (Pharmacia, Uppsala, Sweden) containing 5  $\mu$ g/ml concanavalin A (ConA) and 20 ng/ml of human IL-2 (provided by Ajinomoto Co., Tokyo, Japan) for 24 h. The cells

were then grown for 6 days in AIM-V medium containing 20 ng/ml of human IL-2, and finally stimulated with 10 ng/ml phorbol myristic acetate and 500 ng/ml ionomycin for 8 h.

Poly(A) RNA was prepared from activated PBL using a mRNA isolation kit from Pharmacia and single-stranded cDNA was synthesized with random hexamer oligonucleotides as primers. In brief, 1.0  $\mu$ g of poly(A) RNA was incubated at 42°C for 60 min with RNaseH<sup>-</sup> M-MLV reverse transcriptase (Superscript II; GibcoBRL, Gaithersburg, MD) with 50 ng of d(N)<sub>6</sub> as a primer in a total volume of 20  $\mu$ l. After incubation, a 2  $\mu$ l aliquot of the reaction mixture was diluted with 100  $\mu$ l of PCR buffer (21), and human FasL cDNA was amplified by PCR using a sense primer carrying a 20 nucleotide sequence (CTACAGGACTGAGAAGGT) upstream of the ATG initiation codon and an antisense primer carrying a 20 nucleotide sequence (ACATTCTCGGTGCCTGTAAC) downstream of the TAA termination codon. An XbaI recognition sequence (GCTCTAGA) was added to the 5' end of each primer. The reaction mixture for PCR contained 100 pmol each of the sense and antisense primers, and the reaction was started by adding 2.5 units of *Thermus aquaticus* DNA polymerase (Taq polymerase). The conditions for PCR were 1.0 min at 94°C, 2.0 min at 55°C and 3 min at 72°C for 20 cycles. The PCR product was digested with XbaI and ligated into pBluescript II.

## Fluorescent in situ hybridization (FISH)

FISH was performed essentially as described (22). In brief, human (pro)metaphase chromosomes were prepared from normal male lymphocytes using the thymidine synchronization, BrdU release technique for the delineation of G-bands. Before hybridization *in situ*, chromosomes were stained in Hoechst 33258 and irradiated with UV. Recombinant  $\lambda$ DNAs ( $\lambda$ HFL4 and 5) carrying human FasL chromosomal region were labeled with biotin-16-dUTP by nick-translation and hybridized to the denatured chromosome on slides at a final concentration of 25 ng/ $\mu$ l in a mixture of 50% formamide, 10% dextran sulfate (Sigma, St Louis, MO), 2 $\times$ SSC, Cot-1 DNA (GibcoBRL; 0.2  $\mu$ g/ $\mu$ l), sonicated salmon sperm DNA (2  $\mu$ g/ $\mu$ l) and *Escherichia coli* tRNA (2  $\mu$ g/ $\mu$ l). The hybridization signals were detected with FITC-avidin (Boehringer, Mannheim, Germany) and chromosomes were counterstained with 1  $\mu$ g/ $\mu$ l propidium iodide. The precise signal position was determined by the delineation of G-banding patterns (22).



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b

20

Met Gln Gln Pro Phe Asn Tyr Pro Tyr Pro Gln Ile Tyr Trp Val Asp Ser Ser Ala Ser Ser Pro Trp Ala Pro
ATG CAG CAG CCC TTC AAT TAC CCA TAT CCC CAG ATC TAC TGG GTG GAC AGC AGT CCC AGC TCT CCC TGG GCC CCT

40  
 . Pro Gly Thr Val Leu Pro Cys Pro Thr Ser Val Pro Arg Arg Pro Gly Gln Arg Arg Pro Pro Pro Pro Pro Pro  
 CCA GGC ACA GTC CTT CCC TGT CCA ACC TCT GTG CCC AGA AGG CCT GGT CAA AGG AGG CCA CCA CCA CCA CCG CCA

60  
 Pro Pro Pro Leu Pro Leu Pro Pro Pro Leu Pro Lys Lys Arg Gly  
 CCG CCA CCA CTA CCA CCT CCG CCG CCG CCC CCA CCA CTG CCT CCA CTA CCG CTG CCA CCC CTG AAC AAG ACA CGG

Asn His Ser Thr Gly Leu Cys Leu Leu Val Met Phe Phe Met Val Leu Val Ala Leu Val Gly Leu Gly Leu Gly	100
AAC CAC AGC ACA GGC CTG TGT CTC CTT GTC ATG TTT TTC ATG GTT CTG GTT CCC TTG GTA GGA TTG GGC CTG GGG	

Met Phe Gln Leu' Phe His Leu Gln Lys Glu Leu Ala Glu Leu Arg Glu  
ATG TTT CAG CTC TTC CAC CTA CAG AAG GAG CTG GCA GAA CTC CGA GAG GTAAAGCTGCCGGCAGACTGCTGTG ... intron 1

120 Ser Thr Ser Gin Met His Thr Ala Ser Ser Leu Glu Lys Gln Ile G  
 ..ATCTTTCTCTTCTGTTTACTAG TCT ACC AGC CAG ATG CAC ACA GCA TCA TCT TTG GAG AAG CAA ATA G GTGAGTCCT

TTTTCGCATGTACAT...intron 2....TATTTTCCTCTCTATGATACAG

Leu Arg Lys Val Ala His Leu Thr G  
CTG AGG AAA GTG GCC CAT TTA ACA G GTCTGTATCTGGAGGTACAGGTGA...intron 3....AAAGCTCCTTTGGATTATTCAG

ly Lys Ser Asn Ser Arg Ser Met Pro Ile Glu Trp Glu Asp Thr Tyr Gly Ile Val ·Leu Leu Ser Gly Val Lys  
GC AAG TCC AAC TCA AGG TCC ATG CCT CTG GAA TGG GAA GAC ACC TAT GGA ATT GTC CTG CTT TCT GGA GTG AAG

Tyr Lys Lys Gly Gly Leu Val Ile Asn Glu Thr Gly Leu Tyr Phe Val Tyr Ser Lys Val Tyr Phe Arg Gly Gln  
TAT AAG AAG GGT GCC CTT GTG ATC AAT GAA ACT GGG CTG TAC TTT GTA TAT TCC AAA GTA TAC TTC CGG GGT CAA

Ser Cys Asn Asn Leu Pro Leu Ser His Lys Val Tyr Met Arg Asn Ser Lys Tyr Pro Gln Asp Leu Val Met Met  
 TCT TGC AAC AAC CTG CCC CTG AGC CAC AAG GTC TAC ATG AGC AAC TCT AAG TAT CCC CAG GAT CTG GTG ATG ATG  
  
 Glu Gly Lys Met Met Ser Tyr Cys Thr Thr Gly Gln Met Trp Ala Arg Ser Ser Tyr Leu Gly Ala Val Phe Asn  
 GAG GGG AAG ATG ATC AGC TAC TGC ACT ACT GGG CAG ATG TGG GCC CGC AGC AGC TAC CTG GGG GCA CTG TTC AAT

Lec Thr Ser Ala Asp His Leu Tyr Val Asn Val Ser Glu Leu Ser Leu Val Asn Phe Glu Glu Ser Gln Thr Phe  
 CTT ACC ACT GCT GAT CAT TTA TAT GTC AAC GTC TCT GAG CTC TCT CTC GTC AAT TTT GAC GAA TGT GAC AGC TTT

280  
 Phe Gly Leu Tyr Lys Leu  
 TTC GGC TTA TAT AAG CTC TAA GAGAAGGACTTGGGATTCTTCCATTATGATTCTTGTGTTACAGGCACCGAGAATGTTGATTCACTGAGG  
 CTCTTCTTACATGCATTGAGGTCAAGTAACAAGACATGAACCAAGTGGACCTTGAGACCACAGGCTCAAAATGTCCTAGCTCTCAACTCACCTAA  
 TGTATGAGCCAGACAATGGAGGAATATGACGGAGAACATAGAACTCTGGCTGCCATGTGAAGAGGGAGAACATGAAAAAGCAGCTACCAGGTG  
 TTCTACACTCATCTTAGTGCCTGAGAGTATTTAGGCACATTGAAAAGGGACACC

**Fig. 1.** The gene structure of human FasL. (a) The gene organization of human FasL. The organization of the human FasL gene is shown schematically. Boxes and the lines between them represent four exons and three introns respectively. The filled areas represent the coding sequence, while the non-coding regions are indicated by open areas. The recognition sites for *Pst*I (P), *Bam*H I (B), *Bgl*II (Bg), *Eco*RI (E) and *Hind*III (H) are shown. (b) The genomic sequence of human FasL. The nucleotide sequence of exons and 5' promoter region of the human FasL is shown with the predicted amino acid sequence for the exons. Amino acids are numbered starting at Met-1. The TATA box is underlined and the primers for PCR are indicated by arrows. These sequence data are available from EMBL/GenBank/DDJB under accession number 411821.

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## Assay of cytotoxic activity

The *Xba*I DNA fragment carrying human or mouse FasL cDNA was inserted into the mammalian expression vector pEF-BOS (23). Monkey COS cells cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS were transfected with the expression plasmid using the DEAE-dextran method (24). Forty eight hours later, the cytotoxic activity of the transfected COS cells was determined as described previously (9). In brief, mouse WR19L and its transformants expressing mouse Fas (W4) (19) or human Fas (WC8A) (25) were labeled with  $^{51}\text{Cr}$ . The  $^{51}\text{Cr}$ -labeled cells ( $1 \times 10^4$ ) were incubated for 4 h at 37°C with the transfected COS cells at various ratios in round-bottomed microtiter plates in a total volume of 200  $\mu\text{l}$ . After incubation, the plates were centrifuged and the radioactivity in 100  $\mu\text{l}$  aliquots of the supernatants was determined. The spontaneous release of  $^{51}\text{Cr}$  was determined by incubating the target cells with medium alone, whereas the maximum release was determined by adding Triton X-100 to a final concentration of 0.1%. The percent specific lysis was calculated as follows: [(experimental  $^{51}\text{Cr}$  release - spontaneous  $^{51}\text{Cr}$  release)/(maximum  $^{51}\text{Cr}$  release - spontaneous  $^{51}\text{Cr}$  release)  $\times$  100]. The spontaneous release of  $^{51}\text{Cr}$  was routinely 8–10% of the maximum release.

The soluble forms of mouse Fas (mFas-Fc) or human Fas (hFas-Fc) were prepared by expressing the hybrid gene consisting of the extracellular region of mouse or human Fas fused to the Fc region of the human Ig heavy chain (IgH) as described previously (10). mFas-Fc and hFas-Fc were purified using Protein A-Sepharose and added to the assay mixture to examine their inhibitory effects.

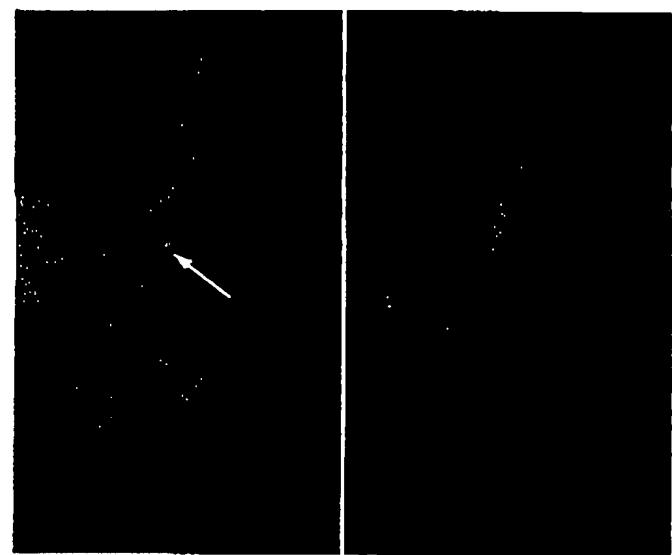
## Results

## Chromosomal gene for human FasL

A human genomic library constructed with human placenta DNA and  $\lambda$ EMBL-3 SP6/T7 vector was screened under low stringency with the 5' or 3' part of rat FasL cDNA as a probe, which resulted in three positive clones ( $\lambda$ HFL4, 5 and 7). Since  $\lambda$ HFL5 gave positive signals with both 5' and 3' probe DNAs, it was further characterized by restriction enzyme mapping and Southern hybridization. The nucleotide sequence of the human FasL genomic region determined after subcloning into pBluescript II is presented in Fig. 1. A comparison of the human genomic DNA sequence with the rat FasL cDNA (9) and human FasL cDNA obtained by reverse PCR (see below) revealed the structural organization of the human FasL gene (Fig. 1). It consists of ~8.0 kb and is split into four exons. All of the splice donor and acceptor sites conformed to the GT-AG rule, and further flanking sequences were in good agreement with favored nucleotide frequencies noticed in other split genes (26). The gene organization of human FasL including the position of introns was the same as that of mouse FasL (11), and similar to that of other members of the TNF family which include TNF- $\alpha$  and LT- $\beta$  (27,28).

## Assignment of human FasL gene to 1q23

Assignment of several (pro)metaphase chromosome spreads localized FasL  $\lambda$ DNAs ( $\lambda$ HFL4 and 5) to human chromosome 1q23. To precisely sublocalize FasL on the region 1q23, 10



**Fig. 2.** FISH of the human FasL gene. (Left) Partial metaphases showing the FITC signals (arrow) on both chromatids of chromosome 1. (Right) The G-banding pattern of the same chromosome, indicating that the FITC signal is at 1q23.3.

prometaphase chromosomes 1 exhibiting twin-spots of FITC signals were visualized through a B-2A filter (Nikon, Tokyo, Japan) and then G-band patterns on the same chromosomes were delineated through a UV-2A filter (Nikon, Tokyo, Japan). As shown in Fig. 2, the signals were sublocalized at 1q23.3 of the long arm of human chromosome 1.

## Primary sequence of the human FasL

Activated rat or mouse splenocytes express FasL mRNA (9,11). To obtain human FasL cDNA, poly(A) RNA was prepared from human PBL activated with Con A and IL-2. The human FasL cDNA was then amplified by reverse PCR using appropriate primers representative of the 5' or 3' non-coding regions of the human FasL gene (Fig. 1). The resultant 970 bp PCR product was inserted into pBluescript II and its nucleotide sequence was determined. The sequence was completely identical to that of exons of the human FasL chromosomal gene and contained a long open reading frame of 843 bp. The reading frame codes for a polypeptide of 281 amino acids with a calculated  $M_r$  of 31,759. As in rat and mouse FasLs (9,11), human FasL is also a type II membrane protein, and it is highly homologous to mouse and rat FasLs. Alignment of their amino acid sequences showed that human FasL has an identity of 76.9 and 75.8% with mouse FasL and rat FasL respectively (Fig. 3). When conservative amino acid replacement is considered as homologous, the similarity of human FasL with mouse FasL and rat FasL increased to 86.2 and 84.0% respectively.

## No species specificity of FasL between human and mouse

The human FasL cDNA was inserted into a mammalian expression vector and introduced into COS cells. As shown in Fig. 4, the COS cells transfected with human FasL cDNA

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hFasL:	MQQPFNYPPQIYWVDSSASSPWA PPGTVLPCPTSVPRPGQR RPPPPPPPLPPPPP <b>P</b>	20	mFasL:	MQQPMNYPCPQIFWVDSSATSS WAPPGSVFCPCPSCGPRGP DQRRPPPPPS-PLPPPS <b>P</b>	40	rFasL:	MOOPVNYP CPOIYWVDSSATSP WAPPGSVFC PSSGPRCPG ORRPPPPPS-PLPPPS <b>P</b>	60
hFasL:	PPLPPPLPLKKRGNHSTGL GLVMPMVLVALVGL OLFHLOKELAELRESTSQ <b>P</b>	80	mFasL:	QPLPLPPLTPLKKKDHN -TNL ALPVWVPMV LVALVGM GICLGM YQLFHLQKELAELREFTNQ <b>P</b>	100	rFasL:	<u>QPPPLPPLSPLKKDN</u> --IE LMLPVDE SMV LVALVGM GICLGM YOLFHLOKELAELREFTNH <b>P</b>	120
hFasL:	MHTASSLEKQIGHPS PPPEKKERKVAH LTGKSNSRSM PLEWEDTYGIV LLSGVKYKKGG <b>P</b>	140	mFasL:	SLKVSSFEKQIANP STPSEKKEPR SVAHLTG NPHSRSI PLEWEDTYG TALISGV KYKKGG <b>P</b>	160	rFasL:	SLRVSSFEKO IANTP SETKK PRSV VAHLTG NPRSRSI PLEWEDTYG TALISGV KYKKGG <b>P</b>	180
hFasL:	LVINETGLYFVY SKVYFRGQSCNN LPLSHK VYMRNSK YPQDLV MMEGK MMSYCT TGQMWA <b>P</b>	200	mFasL:	LVINETGLYFVY SKVYFRGQSCNN QPLNHK VYMRNSK YPEDTL VLMEEK KRLNYC TTGQIWA <b>P</b>	220	rFasL:	<u>LVINEAGLYFVY</u> SKVYFRGQSCNS QPLSHK VYMRNF YPGDLV MEKKLN NYC TTGQIWA <b>P</b>	240
hFasL:	RSSYLGAVFN NLTSADH LYVN VSEL SLVN FEE SQTFF GLYKL <b>P</b>	260	mFasL:	HSSYLGAVFN NLTSADH LYVN ISQLS LIN FEE SKTF FFGLYKL <b>P</b>	280	rFasL:	HSSYLGAVFN LTADH LYVN ISOLS LIN FEE SKTF FFGLYKL <b>P</b>	

Fig. 3. Comparison of the amino acid sequences of human FasL, mouse FasL and rat FasL. The amino acid sequences of human FasL (hFasL), mouse FasL (mFasL) (11) and rat FasL (rFasL) (9) are aligned to give maximal homology by introducing several gaps (-). Numbers indicate the amino acid number for human FasL. The amino acid residues conserved in three species are underlined. The proline residues in the cytoplasmic region of the FasL are shown in bold letters, while the amino acids in the putative transmembrane domain are presented in shadowed letters.

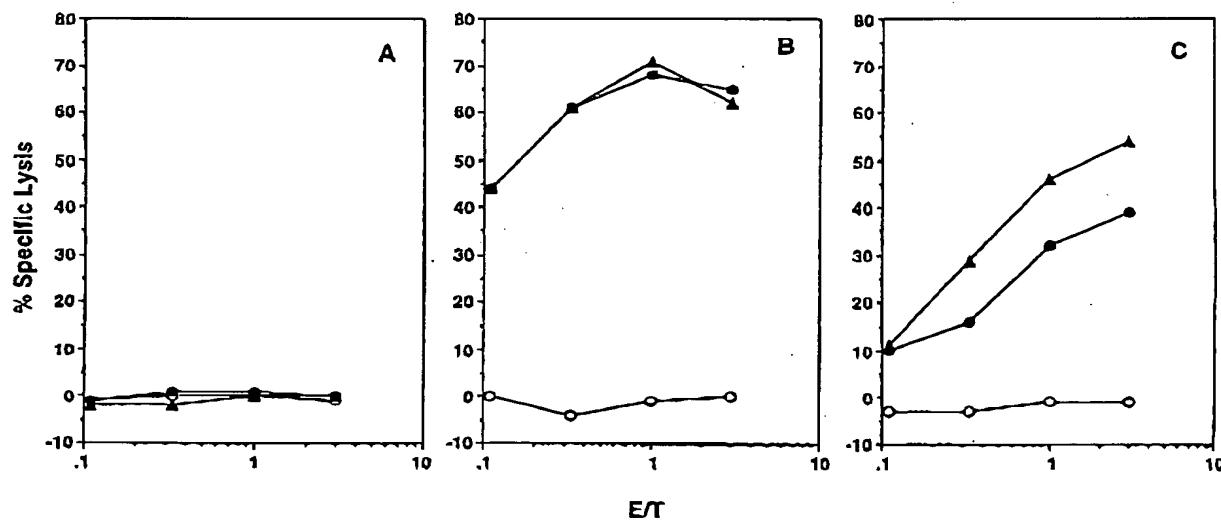
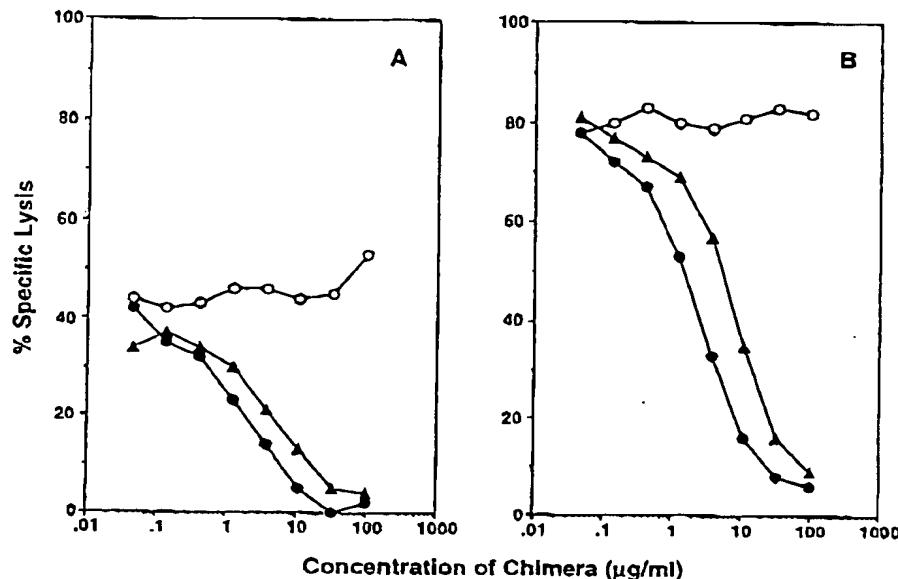


Fig. 4. Cytotoxic activities of human and mouse FasL. COS cells were transfected with pEF-BOS vector (○), or pEF-BOS carrying human FasL (▲) or mouse FasL cDNA (▲). After 48 h, the cytotoxicity of the transfected COS cells was determined using WR19L cells (A), W4 cells (B) and WC8A cells (C) as target cells as described under Methods. The assays were done in duplicate and the values agreed within 10% error. The average values are plotted.

lysed the transformants expressing mouse Fas (W4) or human Fas (WC8A) in a dose-dependent manner. The recombinant human FasL expressed in COS cells did not show any cytolytic activity on parental WR19L cells which hardly express Fas. Similarly, mouse FasL expressed in COS cells lysed both W4

and WC8A cells as efficiently as human FasL. These results indicated that human FasL can bind to mouse as well as human Fas and vice versa. To confirm the lack of species specificity of the Fas system between human and mouse, the soluble forms of human Fas (hFas-Fc) and mouse Fas (mFas-

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**Fig. 5.** Inhibition of the human FasL-induced cytotoxicity by the soluble form of Fas. COS cells were transfected with the human FasL expression plasmid. The cytotoxic activity of the recombinant FasL was then assayed using WC8A cells (A) or W4 cells (B) as target cells at the E/T (effector cells/target cells) ratio of 0.75. The indicated concentrations of the soluble form of human type I TNF receptor (hTNFR $\beta$ -Fc, ○), human Fas (hFas-Fc, ●) or mouse Fas (mFas-Fc, ▲) were added to the assay mixture. The assays for cytotoxicity were done in duplicate and the average values are plotted. The difference in duplicate was within 20%.

Fc) were prepared as described previously (10). As shown in Fig. 5, both hFas-Fc and mFas-Fc inhibited the human FasL-induced cytolysis of WC8A or W4 cells in a similar dose-dependent manner. On the other hand, the soluble form of human TNF receptor type I (hTNFR $\beta$ -Fc) did not inhibit the human FasL-induced cytotoxicity.

## Discussion

The mouse FasL gene is localized on chromosome 1 and the mouse *gld* mutant carries a mutation in the FasL (11). Here, human FasL gene was mapped on chromosome 1q23.3 which is homologous to the *gld* locus of the mouse, confirming our previous assignment of FasL to the *gld* mutation. Mice homozygous in the *gld* mutation show lymphadenopathy and autoimmune disease. It is possible that similar human diseases such as angioimmunoblastic lymphadenopathy (29) are caused by a loss-of-function mutation in FasL or Fas. Furthermore, the fact that the FasL and Fas mediate apoptosis suggests that these genes function as tumor suppressor genes. T cell lymphoma or other malignant cells should be investigated to determine whether they carry an abnormality in the FasL or Fas gene. The FasL is a member of the TNF family. Accordingly, the chromosomal gene structure of FasL is similar to those of other members of the TNF family such as TNF- $\alpha$  and LT- $\beta$  (27,28). The TNF- $\alpha$  and LT- $\beta$  genes are tandemly arranged near the HLA gene cluster on human chromosome 6p21 (28), whereas the human FasL gene is on chromosome 1q23. Near the FasL gene, there is a gene cluster for CD1 (thymocyte antigen) related to HLA class I (30). These results indicate that a large region of chromosome

including the HLA gene cluster and the ancestral gene for TNF/FasL is duplicated, and that one of them has translocated to another chromosome.

Members of the TNF family have no species specificity between human and mouse (31,32), except that human TNF- $\alpha$  cannot bind mouse type II TNF receptor (33). Here, we showed that the FasL also has no species-specificity between human and mouse. Both human and mouse FasLs induced apoptosis in cells expressing either mouse or human Fas with the same efficiency. Although the overall amino acid sequence of FasL is highly conserved (76.9% identity) between human and mouse (Fig. 3), the similarity between human and mouse Fas is much less pronounced (49.3% identity). More conservation of the ligand than the receptor sequence is also observed in other members of the TNF family.

Among members of the TNF family, the FasL has a rather long N-terminal intracellular region (9,11). We remarked that in rat FasL, this region is very rich in proline residues (9). This property can be seen in human and mouse FasL, but not in other members of the TNF family. The proline-rich sequence has been found in various proteins and binds to the SH3 (*src* homology region 3) domain (34). The consensus sequence for the SH3 binding site was proposed to be XPXXPPP $\psi$ XP ( $\psi$  represents a hydrophobic amino acid) (34). This sequence can be found in human, mouse and rat FasL. The SH3 domains play important roles in mediating specific protein-protein interactions, specifically in the cytoskeleton (35). Most cytokines are soluble proteins and the binding of cytokines to their receptor induces a specific signal in the target cells. Afterwards, the cytokine-receptor complex is internalized in the target cells and cytokines are degraded in the cells. In

## Molecular cloning of human FasL 1573

hFasL:  
mFasL:

ATTCTCCTGTAGCTG-GGAGCAGTTCAC  
ACACCCAG-AGCTGCAGAAGAGCTAAT

hFasL: ACTAAC-**AGGGCTATA**CCCCATGCTGACCTGCTCTGCAGGATCCCAGGAAGGTGAGCAT  
mFasL: GTCCTCAAGGGTAT---CCAGCGCTGACTTGCTGAGTTGGACCTCAGGCAGGCAAGCCT  
-350

hFasL: AGCCTACTAACCTGTTGGTAGCACAGCGACAGCAACTGAGGCCCTGAAGGCT-GTTAT  
mFasL: GGTTCACCAGCCTCTCAGTTAGCACAGAGACGCCATTGAACTTCGAAGACTTGTGCGT  
-300

hFasL: CAGAAA**ATTGTGGGCGGAA**ACTTCCAGGGTTGCTGAGCTCTT-GAGGCTCTCAG  
mFasL: CAGAA**ATTCTGGGCGGAA**ACTTC**CTGGGTT**-GCTGTGAGCTTTGGAGGCTCTCAG  
-250 SP-1 NF- $\kappa$ B

hFasL: CTTCAGCTGCAAAGTGA**GAGTGGGTGTTCTT**GAGAACGAGAATCAGAGA-GAGAG-AGAT  
mFasL: CTTCAGATGC-AAGTGA**GAGTGGGTGTC**CAGAGAACGAAA---GAGAAGAGAACAG--  
IRF-1 -150

hFasL: AGAGAAAGAGAAAGACAGAGGTGTTCCCTAGCTATGAAACTCTATAAGAGAGATCCA  
mFasL: -GAGAA-----AGGTGTTCCCTGACTCGGAAACTTTATAAGAAAACCTTA  
TATA -100

hFasL: GCTTGCCTCCTCTTGAGCAGTCAGCAACAGGGTCCCGTCCTGACACCTCAGCCTCT---  
mFasL: GCTT----CTCTGGAGCAGTCAGCGTCAGAGTTCTGCTCCTGACACCTGAGTCTCCTCC  
-50

hFasL: ACAGGACTGAGAAGAAGTAAA**ACCGTTGCTGGGCTGGCCTGACTCACCA**GCTGCCATG  
mFasL: ACAAGGCTGTGA-GAAG-GAAACCCCTTC**CTGGGCTGGG**-----TGCCATG

Fig. 6. Comparison of the 5' flanking regions of human and mouse FasL genes. In aligning the sequences, gaps (-) were introduced to obtain maximal homology. On the nucleotide sequence, number 1 indicates the ATG initiation codon. The nucleotide residues conserved between human and mouse FasL genes are shown by bold letters. The binding sites for SP-1, NF- $\kappa$ B and IRF-1, and the TATA box are underlined.

contrast, the FasL is a type II membrane protein and expressed in activated T cells. The interaction of FasL on the effector cells with Fas on the target cells induces an apoptotic signal in the target cells. What happens to the FasL on the effector cells remains unknown. Although it is possible that the FasL is cleaved from the effector cells and internalized into the target cells, there are other possibilities. The FasL may be down-regulated in the effector cells or it may be phagocytosed by the target cells as found in photoreceptor development in *Drosophila* (36). The proline-rich sequence in the cytoplasmic region of the FasL may play a role in its down-regulation. In any case, it will be of interest to examine whether the SH3 domain can bind this proline-rich region of FasL or not.

The FasL is expressed in activated T lymphocytes. As shown in Fig. 6, the promoter region of human and mouse FasL genes is significantly conserved up to 200 bp from the TATA box. In this region, we can find several cis-regulatory elements for transcription factors. At 90 (for human) or 70 bp (for mouse) upstream of the TATA box, there is a binding site for IRF-1. The element for IRF-1, originally identified in IFN genes as elements required for induction of IFN by virus, can be found in the promoter of various IFN-responsive genes (37). Furthermore, since activation of T cells greatly induces the expression of IRF-1 (37) and IRF-1-deficient mice show some defect in T cell development (38), a role for IRF-1 in T-cells has been suggested. It would be of interest to examine whether IRF-1 or its related factors such as IRF-2 or IRF-3 regulate the FasL gene expression in T cells. The promoter

of the FasL gene also contains SP-1 and NF- $\kappa$ B binding sites. SP-1 is a rather ubiquitous factor and it is found in the promoter of many house-keeping genes. On the other hand, many inducible genes for lymphokines and monokines including the TNF gene carry NF- $\kappa$ B or its related NFAT element (39,40), which is indispensable for the induction of the gene. It is likely that the NF- $\kappa$ B element in the FasL gene also plays an important role in the induction of this gene.

In conclusion, we presented the amino acid sequence of human FasL, which is a death factor expressed in CTL. FasL may be involved in various human diseases mediated by autoreactive cytotoxic T cells. The human FasL cDNA would be an important tool with which to elucidate the pathological role of FasL in human disease. FasL seems to be transiently expressed in activated T cells. Using the FasL promoter sequence isolated here, the kinds of signals activate the FasL gene can be examined.

#### Acknowledgements

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#### Abbreviations

Con A	concanavalin A
CTL	cytotoxic T lymphocytes
FasL	Fas ligand
FISH	fluorescent <i>in situ</i> hybridization

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gl <sup>d</sup>	generalized lymphoproliferative disease
lpr	lymphoproliferation
LT	lymphotoxin
NGF	nerve growth factor
PBL	peripheral blood lymphocytes
PCR	polymerase chain reaction
TNF	tumor necrosis factor

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## Exhibit 3



(3)

PubMed Nucleotide Protein Genome Structure PMC Taxonomy OMIM Books

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VERSION AAC50124.1 GI:595431  
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ORGANISM Homo sapiens  
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Mammalia: Eutheria: Primates: Catarrhini: Hominidae: Homo.  
REFERENCE 1 (residues 1 to 281)  
AUTHORS Takahashi, T., Tanaka, M., Inazawa, J., Abe, T., Suda, T. and Nagata, S.  
TITLE Human Fas ligand: gene structure, chromosomal location and species specificity  
JOURNAL Int. Immunol. 6 (10), 1567-1574 (1994)  
MEDLINE 95127560  
PUBMED 7826947  
REFERENCE 2 (residues 1 to 281)  
AUTHORS Nagata, S.  
TITLE Direct Submission  
JOURNAL Submitted (06-JUL-1994) Shigekazu Nagata, Molecular Biology, Osaka Bioscience Institute, 4-6-2 Furuedai, Suita, Osaka, 565 Japan  
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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C07H 21/04, C12N 15/19, 15/63, C07K 14/52, 16/24</b>		A1	(11) International Publication Number: <b>WO 95/18819</b> (43) International Publication Date: <b>13 July 1995 (13.07.95)</b>
(21) International Application Number: <b>PCT/US95/00362</b> (22) International Filing Date: <b>6 January 1995 (06.01.95)</b>		(81) Designated States: AU, CA, JP, NZ, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(30) Priority Data: 179,138 7 January 1994 (07.01.94) 190,559 1 February 1994 (01.02.94)		US	Published <i>With international search report.</i>
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(72) Inventor: GOODWIN, Raymond, G.; 3322 8th Avenue West, Seattle, WA 98119 (US).			
(74) Agents: ANDERSON, Kathryn, A. et al.; Immunex Corporation, 51 University Street, Seattle, WA 98101 (US).			
(54) Title: LIGAND THAT BINDS FAS ANTIGEN			
(57) Abstract			
Novel human and murine proteins designated Fas ligand (Fas-L) bind to the cell surface protein known as Fas antigen. DNA sequences, expression vectors and transformed host cells useful in producing Fas-L polypeptides are provided, along with antibodies immunoreactive with Fas-L.			

## SEQUENCE LISTING

## 5 (1) GENERAL INFORMATION:

(i) APPLICANT: GOODWIN, Raymond G.

(ii) TITLE OF INVENTION: Ligand That Binds Fas Antigen

10 (iii) NUMBER OF SEQUENCES: 5

## (iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Immunex Corporation

(B) STREET: 51 University Street

(C) CITY: Seattle

(D) STATE: WA

(E) COUNTRY: US

(F) ZIP: 98101

## 20 (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: Apple Macintosh

(C) OPERATING SYSTEM: Apple 7.1

25 (D) SOFTWARE: Microsoft Word, Version 5.1a

## (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE: 06-JAN-1995

(C) CLASSIFICATION:

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(A) APPLICATION NUMBER: US 08/179,138

(B) FILING DATE: 07-JAN-1994

## 35 (viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Anderson, Kathryn A.

(B) REGISTRATION NUMBER: 32,172

(C) REFERENCE/DOCKET NUMBER: 2805-WO

40

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(D) TOPOLOGY: linear

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WO 95/18819

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<b>15</b> AAG CTC TAAGAGAAGC ACTTTGGAT TCTTTCCATT ATGATTCTTT GTTACAGGCA Lys Leu 280	985
CCGAGAACGT TGTATTCACT GAGGGTCTTC TTACATGCAT TTGAGGTCAA GTAAGAACAC <b>20</b> ATGAACCAAG TGGACCTTGA GACCACAGGG TTCAAAATGT CTGTAGCTCC TCAACTCACC TAATGTTTAT GAGCCAGACA AATGGAGGAA TATGACGGAA GAACATAGAA CTCTGGCTG CCATGTGAAG AGGGAGAACG ATGAAAAAGC AGCTACCAAG TGTTCTACAC TCATCTTAGT <b>25</b> GCCTGAGAGT ATTAGGCAG ATTGAAAAGG ACACCTTTA ACTCACCTCT CAAGGTGGC CTTGCTACCT CAAGGGGAC TGTCTTCAG ATACATGGTT GTGACCTGAG GATTTAAGGG <b>30</b> ATGGAAAAGG AAGACTAGAG GCTTGCATAA TAAGCTAAAG AGGCTGAAAG AGGCCAATGC CCCACTGGCA GCATCTTCAC TTCTAAATGC ATATCCTGAG CCATCGGTGA AACTAACAGA TAAGCAAGAG AGATGTTTG GGGACTCATT TCATTCTAA CACAGCATGT GTATTTCCAG <b>35</b> TCGAATTGTA GGGGTGTGTG TGTGTGTGTG TGTGTGTGTG TGTGTATGAC TAAAGAGAGA ATGTAGATAT TGTGAAGTAC ATATTAGGAA AATATGGTT GCATTTGGTC AAGATTTGA <b>40</b> ATGCTTCCTG ACAATCAACT CTAATAGTGC TTAAAAATCA TTGATTGTCA GCTACTAATG ATGTTTCCT ATAATATAAT AAATATTTAT GTAGATGTGC ATTTTGTGA AATGAAAACA TGTAAATAAA AGTATATGTT AGGATACAAA TAAAAAAAAA AAAAAAAAAA AAAAAAAAAA <b>45</b> AAAAAAACCG GAATC	1045 1105 1165 1225 1285 1345 1405 1465 1525 1585 1645 1705 1765 1825 1841

→ (2) INFORMATION FOR SEQ ID NO:2:

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	(A) LENGTH: 281 amino acids
	(B) TYPE: amino acid
	(D) TOPOLOGY: linear
<b>55</b>	(ii) MOLECULE TYPE: protein
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:
<b>60</b>	Met Gln Gln Pro Phe Asn Tyr Pro Tyr Pro Gln Ile Tyr Trp Val Asp 1 5 10 15

	Ser Ser Ala Ser Ser Pro Trp Ala Pro Pro Gly Thr Val Leu Pro Cys			
	20	25	30	
5	Pro Thr Ser Val Pro Arg Arg Pro Gly Gln Arg Arg Pro Pro Pro Pro			
	35	40	45	
	Pro Pro Pro Pro Leu Pro Pro Pro Pro Pro Pro Pro Pro Leu Pro			
	50	55	60	
10	Pro Leu Pro Leu Pro Pro Leu Lys Lys Arg Gly Asn His Ser Thr Gly			
	65	70	75	80
	Leu Cys Leu Leu Val Met Phe Phe Met Val Leu Val Ala Leu Val Gly			
15	85	90	95	
	Leu Gly Leu Gly Met Phe Gln Leu Phe His Leu Gln Lys Glu Leu Ala			
	100	105	110	
20	Glu Leu Arg Glu Ser Thr Ser Gln Met His Thr Ala Ser Ser Leu Glu			
	115	120	125	
	Lys Gln Ile Gly His Pro Ser Pro Pro Glu Lys Lys Glu Leu Arg			
	130	135	140	
25	Lys Val Ala His Leu Thr Gly Lys Ser Asn Ser Arg Ser Met Pro Leu			
	145	150	155	160
	Glu Trp Glu Asp Thr Tyr Gly Ile Val Leu Leu Ser Gly Val Lys Tyr			
30	165	170	175	
	Lys Lys Gly Gly Leu Val Ile Asn Glu Thr Gly Leu Tyr Phe Val Tyr			
	180	185	190	
35	Ser Lys Val Tyr Phe Arg Gly Gln Ser Cys Asn Asn Leu Pro Leu Ser			
	195	200	205	
	His Lys Val Tyr Met Arg Asn Ser Lys Tyr Pro Gln Asp Leu Val Met			
	210	215	220	
40	Met Glu Gly Lys Met Met Ser Tyr Cys Thr Thr Gly Gln Met Trp Ala			
	225	230	235	240
	Arg Ser Ser Tyr Leu Gly Ala Val Phe Asn Leu Thr Ser Ala Asp His			
45	245	250	255	
	Leu Tyr Val Asn Val Ser Glu Leu Ser Leu Val Asn Phe Glu Glu Ser			
	260	265	270	
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	275	280		

## (2) INFORMATION FOR SEQ ID NO:3:

55       (i) SEQUENCE CHARACTERISTICS:  
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           (B) TYPE: amino acid  
           (C) STRANDEDNESS: single  
           (D) TOPOLOGY: linear

60       (ii) MOLECULE TYPE: protein

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## The Fas Death Factor

Shigekazu Nagata\* and Pierre Golstein

Fas ligand (FasL), a cell surface molecule belonging to the tumor necrosis factor family, binds to its receptor Fas, thus inducing apoptosis of Fas-bearing cells. Various cells express Fas, whereas FasL is expressed predominantly in activated T cells. In the immune system, Fas and FasL are involved in down-regulation of immune reactions as well as in T cell-mediated cytotoxicity. Malfunction of the Fas system causes lymphoproliferative disorders and accelerates autoimmune diseases, whereas its exacerbation may cause tissue destruction.

Homeostasis of multicellular organisms is controlled not only by the proliferation and differentiation of cells but also by cell death (1). The death of cells during embryogenesis, metamorphosis, endocrine-dependent tissue atrophy, and normal tissue turnover is called programmed cell death. Most of programmed cell death proceeds by apoptosis, a process that includes condensation and segmentation of nuclei, condensation and fragmentation of the cytoplasm, and often extensive fragmentation of chromosomal DNA into nucleosome units.

Apoptosis in vertebrate development often occurs by default when cells fail to receive the extracellular survival signals needed to suppress an intrinsic cell suicide program (2); the survival factors can be produced by neighboring cells of a different type (a paracrine mechanism), or of the same type (an autocrine mechanism). In contrast, in the immune system there are situations where cells actively kill other cells; for example, cytotoxic T lymphocytes (CTLs) or natural killer (NK) cells induce apoptosis in their targets such as virus-infected cells or tumor cells (3). In these cases,

an effector molecule expressed at the surface of CTLs or NK cells or a soluble cytokine produced by these effector cells is thought to be responsible for target cell death.

Molecular and cellular characterization of Fas, a cell surface protein recognized by cytotoxic monoclonal antibodies, revealed its role as a receptor for a Fas ligand (FasL) (4). When FasL binds to Fas, the target cell undergoes apoptosis. Spontaneous mutations for Fas and FasL have been identified in mice, and from the phenotypes of these mutants and from studies on mechanisms of cytotoxicity, it was concluded that the Fas-FasL system is involved not only in CTL-mediated cytotoxicity but also in down-regulation of immune responses. In this article, we summarize current knowledge on Fas and FasL and discuss their physiological and pathological roles in the immune system.

### Fas and Fas Ligand

In 1989 two groups independently isolated mouse-derived antibodies that were cytolytic for various human cell lines (5, 6). The cell surface proteins recognized by the antibodies were designated Fas and APO-1, respectively. The antibody to Fas (anti-Fas) was an immunoglobulin M (IgM) antibody, whereas the antibody to APO-1 was classified as IgG3. The Fas complementary DNA

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(cDNA) was isolated by expression cloning from a cDNA library of human KT-3 lymphoma cells which abundantly express Fas (7). Human Fas consists of 325 amino acids with a signal sequence at the NH<sub>2</sub>-terminus and a membrane-spanning region in the middle of the molecule, indicating that Fas is a type I membrane protein.

The function of Fas was assessed by the establishment of mouse cell transformants that constitutively expressed human Fas. When the transformed cells were treated with the antibody to human Fas, the cells died by apoptosis within 5 hours, which indicated that Fas can transduce an apoptotic signal and that anti-Fas works as an agonist. The subsequent purification of human APO-1 antigen and molecular cloning of its cDNA established the identity of APO-1 and Fas (8).

The structure of Fas indicated that it belongs to the tumor necrosis factor (TNF) and nerve growth factor (NGF) receptor family (Fig. 1A) (7-9). This family includes two TNF receptors (TNF-R1 and TNF-R2),

the low-affinity NGF receptor, CD40, OX40, CD27, 4-1BB, and CD30 (10). The extracellular regions of members of this family consist of three to six cysteine-rich domains. The amino acid sequence of the extracellular region is relatively conserved, whereas the cytoplasmic region is not, except for some similarity between Fas and TNF-R1 (7, 8). Subsequent mutational analyses of Fas and TNF-R1 indicated that the cytoplasmic domain (about 70 amino acids) conserved between Fas and TNF-R1 is necessary and sufficient for transduction of the apoptotic signal (11). This domain was therefore designated a death domain.

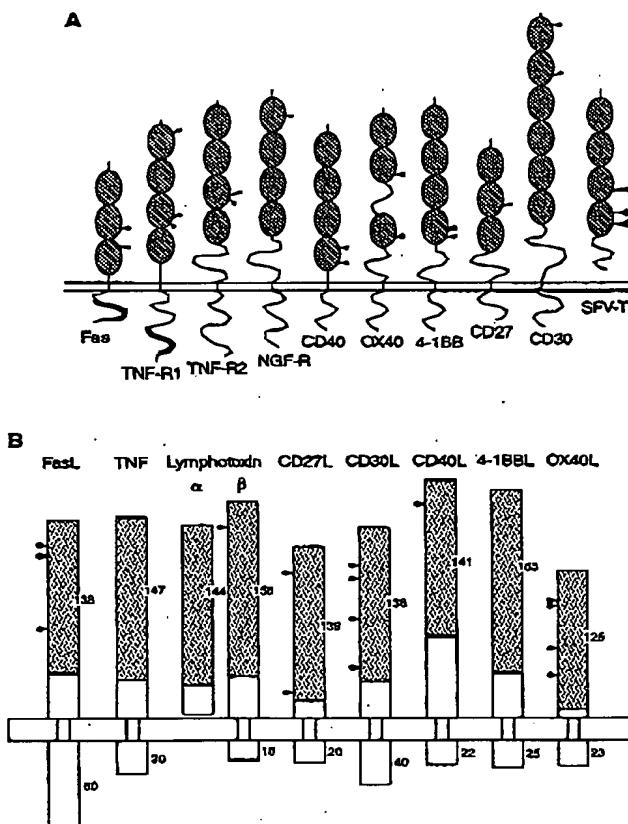
In humans the single Fas gene per haploid genome is located on the long arm of chromosome 10 (12), whereas in the mouse, the gene is on chromosome 19 (9). The human and mouse genes span 12 kb and more than 70 kb, respectively, and both genes comprise nine exons (13). As for the expression patterns of Fas in various tissues and cell lines, there is considerable variability. Many tissues and cell lines weakly ex-

press Fas, but abundant expression was found in mouse thymus, liver, heart, lung, kidney, and ovary (9). Unlike mouse thymocytes human thymocytes only weakly express Fas. In mouse thymocytes, Fas is expressed in almost all populations except for double-negative (CD4<sup>-</sup>CD8<sup>-</sup>) thymocytes (14). Fas is highly expressed in activated mature lymphocytes (5) or lymphocytes transformed with human T cell leukemia virus (HTLV-I), human immunodeficiency virus (HIV), or Epstein-Barr virus (EBV) (15, 16). Some other tumor cells also express Fas, although the expression level is low compared with that of lymphoblastoid cells. The expression of Fas is up-regulated by interferon- $\gamma$  (IFN- $\gamma$ ) in various cell lines (6, 7, 9), or by a combination of IFN- $\gamma$  and TNF- $\alpha$  in human B cells (17), which may explain the enhancement of the cytotoxic activity of anti-Fas by these cytokines (6). The induction mechanism of Fas expression or the promoter elements of the Fas gene have not been intensively studied.

The structure of Fas suggested that Fas was a receptor for an unknown cytokine. In 1993, Rouvier et al. (18) reported on a subline from a cytotoxic T cell hybridoma (PC60) between a mouse CTL cell line and a rat lymphoma. The subline (PC60-d10S, d10S for short) could kill target cells expressing Fas but not target cells which did not express Fas. Soluble forms of Fas (Fas-Fc) and TNF receptor (TNFR-Fc) were constructed by fusing their extracellular regions to human IgG (19). The Fas-Fc but not TNFR-Fc inhibited the CTL activity of d10S cells, indicating that the d10S cells expressed a FasL and that this FasL played a major role in d10S-mediated cytotoxicity. The FasL on d10S cells could be stained by biotinylated Fas-Fc, and a subline of the d10S cells (d10S16), which expressed ~100 times more FasL than did the original d10S cells, was then established by repeated fluorescence-activated cell sorting. The d10S16 cells showed about 100 times more CTL activity against the Fas-expressing cells than did the d10S cells.

FasL was purified to homogeneity from the solubilized membrane fraction of d10S16 cells by means of affinity-chromatography with Fas-Fc (19). The purified FasL was a protein with a molecular weight ratio of ~40,000, and it showed strong cytotoxic activity against Fas-expressing cells. Rat FasL cDNA was then cloned by expression cloning from the d10S subline with Fas-Fc (20), and mouse and human FasL cDNAs were subsequently cloned by cross-hybridization (21, 22). FasL has no signal sequence at the NH<sub>2</sub>-terminus, but it has a domain of hydrophobic amino acids in the middle of the molecule, indicating that it is a type II membrane protein with the COOH-terminal region outside the cell. Mouse and human

**Fig. 1. TNF and its receptor family.** (A) The TNF and NGF receptor family. Members of the TNF-NGF receptor family are schematically shown. These include Fas, TNF-R1, TNF-R2, NGF receptor, B cell antigen CD40, T cell antigens OX40, 4-1BB, and CD27. Hodgkin's lymphoma antigen CD30, and the soluble protein coded by Shope fibroma virus (SPV-T2). The striped regions represent cysteine-rich subdomains; each member of the family contains three to six of them. The death domains (about 80 amino acids) in the cytoplasmic regions of Fas and TNF-R1, which have some similarity, are shown as bold lines. The symbol — indicates an N-glycosylation site. [Reproduced from (4), S. Nagata, with permission from Academic Press.] (B) The TNF family. Members of the TNF family are schematically shown. These include Fas ligand, TNF, the  $\alpha$  and  $\beta$  subunits of lymphotoxin, CD27 ligand, CD30 ligand, CD40 ligand, 4-1BB ligand, and OX40 ligand. These members are type II membrane proteins, except for the  $\alpha$  subunit of lymphotoxin which is a secretory protein. The shaded portion of each member is the extracellular region which shows significant similarity (25 to 30% identity) among the members. The number of the amino acids in the homologous region and the cytoplasmic region are indicated. The symbol — indicates an N-glycosylation site.



## APOPTOSIS: ARTICLES

FasLs are 76.9% identical at the amino acid sequence level, and they are functionally interchangeable. A stretch of about 150 amino acids in the extracellular region of the FasL has significant homology to the corresponding region of other members of the TNF family which includes TNF, lymphotoxin (LT), CD40 ligand, CD27 ligand, CD30 ligand, and OX40 ligand (Fig. 1B). Expression of recombinant FasL on the cell surface of fibroblast-like COS cells was sufficient to induce apoptosis in Fas-expressing target cells within a few hours (20), indicating that FasL is a death factor and Fas is its receptor (4).

A single FasL gene is located on human and mouse chromosome 1 (21, 22) in the neighborhood of the OX40 ligand gene, another member of the TNF family (23). The FasL gene comprises five exons, and its organization is similar to those of TNF- $\alpha$  and LT- $\beta$ . Among the various cell lines examined, only activated T cell lines expressed FasL (24). FasL messenger RNA (mRNA) was not detected in B cells or macrophage, fibroblast, endothelial, or thymic stroma cell lines. The exception was the testis, where abundant FasL expression was found in mouse and rat (20), but interestingly, not in human (25). The expression of FasL in T cells could be rapidly induced by activation with phorbol 12-myristate 13-acetate (PMA) and ionomycin or T cell receptor engagement (24). Herbimycin A and genistein (protein tyrosine kinase inhibitors) as well as cyclosporin A (an inhibitor of the calmodulin-dependent protein phosphatase calcineurin) inhibited the induction of FasL expression. It is likely that some tyrosine kinases and calcineurin are involved in the activation of the FasL gene through the T cell receptor, as has been found for the induction of other lymphokine genes. The nuclear factor- $\kappa$ B (NF- $\kappa$ B) or interferon regulatory factor-1 (IRF-1) element found in the promoter region of the FasL gene may be responsible for this induction (21).

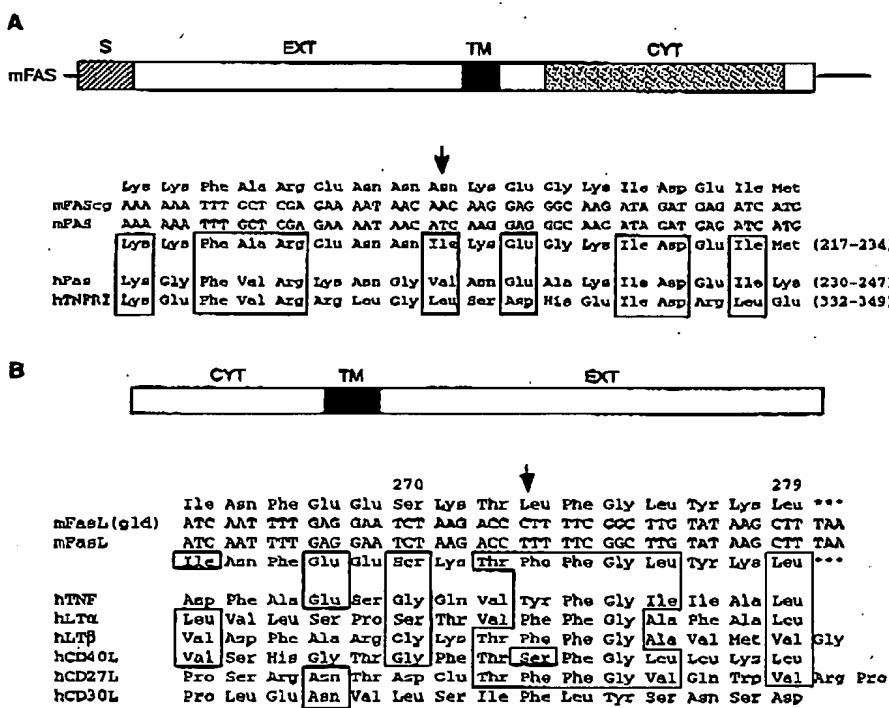
### A Death Signal from Fas

Signaling by means of Fas leads to apoptotic cell death, with characteristic cytoplasmic and nuclear condensation and DNA fragmentation (5, 7, 20, 26, 27). Triggering this pathway requires the cross-linking of Fas either with antibodies to Fas (28), with cells expressing FasL, or with purified FasL (19). Similar to TNF, the soluble form of FasL has a trimeric structure in solution (29). Therefore, it is likely that the cross-linking of Fas molecules, rather than just their engagement, leads to further signaling within the cell.

Cell death via Fas does not require the presence of a nucleus or DNA fragmentation. Cells enucleated with cytochalasin B

undergo apoptosis (that is, the characteristic cytoplasmic lesions appear) when triggered by the Fas-based mechanism with anti-Fas or with cytotoxic T cells (27). This is in line with the fact that a nucleus is not required in other apoptotic death systems, such as staurosporin-induced apoptosis (30). These results, as well as others showing the death-inducing ability of cytoplasmic extracts on isolated nuclei (31), indicate that "nuclear death" is only secondary to essential cytoplasmic death events. What are these cytoplasmic death events and how are they related to the first irreversible step of cell death? The answers are not better known in the case of death via Fas than in the case of death induced by other means. Answers to these questions may provide a biochemical definition of cell death and be of considerable practical importance.

Additional results showed that the Fas-triggered pathway to death is independent of extracellular  $Ca^{2+}$  (18, 32) and does not require macromolecular synthesis (6, 7, 33–35). As for many other death pathways, the cellular background plays an essential role in the interpretation and modulation of the Fas-originating signal. Thus, cell sensitivity or resistance involves factors other than just the level of expression of Fas (26, 33, 36, 37). The molecular basis underlying this variability in response may depend on which intracellular molecules are available to bind given segments of the Fas intracytoplasmic region, or depend on other molecules such as Bcl-2 and related proteins that modulate cell death (38). In fact, the Fas-transduced cell death is partially inhibited by overexpression of Bcl-2 (39), but it is completely inhibited by coexpression of



**Fig. 2. Mutations in Fas and FasL in lpr and gld mice.** (A) A point mutation in the cytoplasmic region of the Fas gene of lpr mice. The upper panel shows the structure of Fas. S, signal sequence; EXT, extracellular region; TM, transmembrane region; CYT, cytoplasmic region. The Fas death domain, which has similarity with that of TNF-R<sup>I</sup>, is shown as the shaded area. The lower panel shows the nucleotide sequence and the predicted amino acid sequence of the wild-type (mFAS) and the mutant Fas (mFAS<sup>gld</sup>). The amino acid sequence of the corresponding region of human Fas (hFas) and TNF-R<sup>I</sup> (hTNFR<sup>I</sup>) are also shown. Sets of three identical or homologous amino acid residues at one aligned position are boxed. The arrow indicates the position of the mutation in lpr mice. [Reproduced from (57) with permission from the author and *Nature*, copyright Macmillan Magazines.] (B) A point mutation in the extracellular region of FasL of gld mice. The upper panel shows the structure of FasL. The lower panel shows the nucleotide and amino acid sequences of the mutant FasL (mFasL<sup>gld</sup>) and wild type (mFasL). The arrow indicates the position of the mutation in FasL of gld mice. Amino acid sequences of the corresponding region of the other members of the TNF family (TNF, LT- $\alpha$ , LT- $\beta$ , CD40L, CD27L, and CD30L) are also shown. The amino acids of favored substitutions in more than four members are boxed. [Reproduced from (22), T. Takahashi et al., with permission from the author and *Cell*, copyright Cell Press.]

Bcl-2 and its binding protein BAG-1 (40). It is not yet known whether molecules such as interleukin-1 converting enzyme (ICE) protease and Cdc2 kinase, which have been implicated in some cell death systems (41), play a role in Fas-induced cell death.

Because Fas and TNF-R1 have homologous cytoplasmic death domains, one might expect that death transduced by means of one or the other of these surface receptors would have similar characteristics. This may not be the case. Unlike TNF-R1-transduced death in the same type of cells, Fas-transduced death is not blocked by manganese superoxide dismutase (MnSOD), metallothionein, plasminogen activator type 2, A20, or mitochondrial inhibitors (37, 42), and it does not activate the transcription factor NF- $\kappa$ B. The latter observation in particular is not completely consistent with indications that it is the death domain of TNF-R1, homologous to that of Fas, which triggers the acidic sphingomyelinase that leads to activation of NF- $\kappa$ B (43). Moreover, the Fas-induced cell death is quicker than that induced by TNF-R1 (44).

Why do these similar receptors transduce death pathways with such different characteristics? One possibility, still unsubstantiated, is that other parts of the cytoplasmic domains of these receptors generate other signals that modulate the main cell death signal. Indeed, distinct signals can originate from distinct parts of the TNF-R1 cytoplasmic region (43). Recently, cytosolic molecules have been identified that can associate with TNF-R2 and CD40, which are other members of the TNF-NGF receptor family (45). It would be interesting to examine whether these cytosolic molecules or related molecules can associate with Fas or TNF-R1.

A fast-developing field of research deals with the possibility that Fas signals by means of the complex lipid ceramide. Ceramide is one of the products that results from the breakdown by sphingomyelinases of sphingomyelin, a sphingosine-fatty acid-phosphorylcholine molecule found in the plasma membrane and cytoplasm. The involvement of ceramide in the Fas death pathway has been suggested by the following experiments. When various Fas-bearing cells were incubated with anti-Fas for 5 min, acidic sphingomyelinase activity was detected, there was partial hydrolysis of sphingomyelin, and the amount of ceramide increased. Cell-permeable synthetic C2-ceramide itself, when added to the culture medium, was able to induce apoptosis in less than 3 hours (46). The addition of sphingomyelinase or ceramide extracellularly led to the induction of cell death with DNA fragmentation and apoptotic morphology (47). Downstream signaling by ceramide might take place by means of a ceramide-activated protein ki-

nase (48), which could account in part for the phosphorylations observed within 1 min after antibody-mediated Fas ligation on Jurkat cells; protein kinase inhibitors block the resulting DNA fragmentation and cell death (49).

### The *lpr* and *gld* Mutations

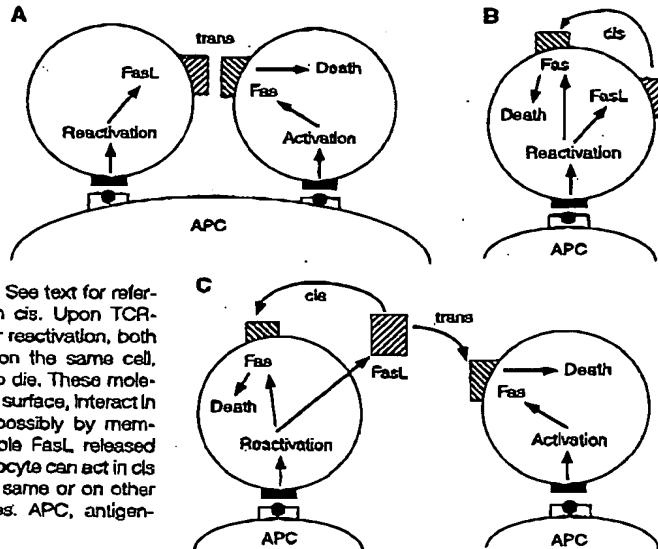
The mouse spontaneous mutants *lpr* (lymphoproliferation) and *gld* (generalized lymphoproliferative disease) carry autosomal recessive mutations (50) on mouse chromosome 19 and 1, respectively (51). MRL *lpr/lpr* and MRL *gld/gld* mice develop lymphadenopathy and splenomegaly and produce large quantities of IgG and IgM antibodies including anti-DNA and rheumatoid factor (52). They develop nephritis or arthritis and die at around 5 months of age. The allelic mutation (*lpr*<sup>-/-</sup>) in the *lpr* locus (53), distinct from the original *lpr* mutant, causes a weak *lpr* phenotype in double heterozygotes with *gld* (*lpr*<sup>-/-</sup>/*+*, *gld*/*+*), in addition to the *lpr* phenotype of its homozygous mutation. The other strains of mice carrying *lpr* or *gld* mutations develop lymphadenopathy and splenomegaly, but not nephritis or arthritis (54). Because the wild-type MRL mice develop weak and delayed autoimmune disease, it is likely that *lpr* and *gld* mutations accelerate or worsen the autoimmune disease rather than induce it.

The lymphocytes that accumulate in the lymph nodes and spleen in *lpr* or *gld* mice are arrested at the G<sub>0</sub>-G<sub>1</sub> stage of the cell cycle and express the T cell marker Thy-1 and the B cell marker B-220 (52). These cells express a rearranged T cell receptor but not a rearranged IgG gene. Because neonatal thymectomy prevents the accu-

mulation of lymphocytes, it seems that it is T cells that accumulate in the mutants. However, the CD4 and CD8 antigens, which are usually expressed on mature T cells, are not expressed on the lymphocytes that accumulate in *lpr* or *gld* mice. The CD4 and CD8 gene loci are demethylated in these lymphocytes, and the administration of anti-Thy-1, anti-CD4, or anti-CD8 inhibits development of the lymphadenopathy (52). When mature CD4<sup>+</sup> T cells from *lpr* mice were transplanted into wild-type mice, these T cells became double-negative T cells (55), suggesting that the cells accumulating in *lpr* mice are derived from mature, single-positive (CD4<sup>+</sup>CD8<sup>-</sup> or CD4<sup>-</sup>CD8<sup>+</sup>) T cells.

Although *lpr* and *gld* mutations are non-allelic mutations, they show a similar phenotype. In 1991, Allen et al. (56) carried out a series of bone marrow transplantation experiments among *lpr*, *gld*, and wild-type mice. From these experiments they concluded that *lpr* and *gld* are mutations in genes encoding a pair of interacting proteins: *gld* may affect a soluble or membrane-bound cytokine, whereas *lpr* may affect its receptor. The Fas gene was mapped to a location near the *lpr* locus on mouse chromosome 19 (9). Northern (RNA) hybridization analysis indicated that, in contrast to wild-type mice, *lpr* mice express very little Fas mRNA in the liver and thymus (57). Characterization of the Fas gene in *lpr* mice indicated that an early transposable element (ETn), a variety of mouse endogenous retrovirus, is inserted into intron 2 (58). The ETn carries a polyadenylate signal (AATAAA) on the long terminal repeat (LTR) sequence, which causes premature termination of the Fas RNA transcript.

Fig. 3. A schematic representation of three mechanisms that may be involved in Fas-based immune down-regulation at the lymphocyte level. (A) Fas and its ligand, FasL, may be expressed on different cells, leading to Fas-based death in *trans* through a cytotoxicity-like mechanism. See text for references. (B) "Suicide" in *cis*. Upon TCR-transduced activation or reactivation, both Fas and FasL appear on the same cell, which is then induced to die. These molecules, situated at the cell surface, interact in an unknown manner, possibly by membrane folding. (C) Soluble FasL released from an activated lymphocyte can act in *cis* or *trans*, that is, on the same or on other Fas-bearing lymphocytes. APC, antigen-presenting cell.



Small mRNAs coding for exons 1 and 2 were found in the thymus and liver of *lpr* mice. When the ETn in the Fas gene of *lpr* mice was inserted into the corresponding intron in a mammalian expression vector, the expression efficiency of the vector was reduced to a few percent of that of the original vector.

These results demonstrated that transcription of the Fas gene is impaired in *lpr* mice by an insertion of a transposable element in an intron of the gene. However, this mutation is leaky. Intact Fas mRNA could be found in the thymus and liver of the *lpr* mice at the level of several percent that of the wild-type mouse. The *lpr<sup>-/-</sup>* mice express normal size Fas mRNA at a level similar to that of wild-type mice (57). However, the mRNA carries a T→A point mutation in the Fas cytoplasmic region (Fig. 2A). This mutation changes an isoleucine to an asparagine in the death domain, abolishing the ability of Fas to transduce the apoptotic signal. Together, these results indicate that *lpr* mutations are loss-of-function mutations of the Fas gene. Recent results with transgenic mice, in which the expression of the Fas gene in T cells of *lpr* mice corrected the phenotype (59), confirm this conclusion.

The FasL gene was similarly mapped on

mouse chromosome 1 where the *gld* mutation is localized (22). There is no rearrangement of the FasL gene in the *gld* mice, but there is a point mutation near the COOH-terminus of the coding region (Fig. 2B). This mutation changes a phenylalanine to a leucine in the extracellular region and abolishes the ability of FasL to bind to Fas.

### Fas-Mediated Death of Lymphocytes

As described above, the mouse spontaneous mutations *lpr* and *gld* are loss-of-function mutations of Fas and FasL, respectively. The abnormal accumulation of lymphocytes in *lpr* and *gld* mice suggested that Fas and FasL may be involved in normal lymphocytic death. In the life of lymphocytes, both T cells and B cells normally die at various stages of their development. Precursor T cells originate in the bone marrow and migrate into the thymus, where they mature into single-positive (CD4<sup>+</sup>CD8<sup>-</sup> or CD4<sup>-</sup>CD8<sup>+</sup>) T lymphocytes. The T cells that can interact with self-major histocompatibility complex (MHC) expressed in the thymus are positively selected (positive selection), whereas those that cannot die by apoptosis. On the other hand, T cells that strongly react with self antigen complexed with self-MHC are induced to undergo apoptosis (negative selection) (60). More than 95% of the T cells that immigrate into the thymus die there; the remaining 5% migrate to the peripheral lymphoid organs as mature T lymphocytes (61). In the periphery, the mature T cells again undergo an additional selection process. Those that interacted with the self antigens expressed only in peripheral tissues would die (peripheral clonal deletion) (62). Furthermore, there must be some mechanism in the periphery to eliminate lymphocytes after they have been activated by antigen to ensure that the organism does not fill up with activated lymphocytes (63).

The positive and negative selections in the thymus are apparently normal in *lpr* mice (64), indicating that a Fas-mediated mechanism is unlikely to be involved. On the other hand, several groups showed that peripheral clonal deletion and the elimination of activated T cells are impaired in *lpr* and *gld* mice (65, 66). Apparently, antigenic stimulation first triggers the proliferation of mature T cells, which are later eliminated by apoptosis. In *lpr* or *gld* mice, an antigen can stimulate the proliferation of mature T cells, but the subsequent death process is severely retarded, both in vitro and in vivo. These results indicate that the Fas system is normally involved in both the clonal deletion of autoreactive T cells in peripheral lymphoid organs and the elimination of activated T cells after they have

responded to foreign antigens.

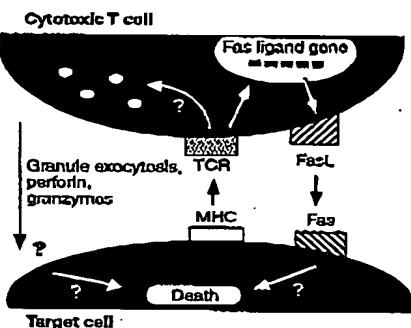
As described below, the Fas system is one of the mechanisms that cytotoxic T cells use to kill infected target cells. A similar cytotoxic mechanism may operate to delete activated T cells. Although mature T cells constitutively express Fas, activation by antigens up-regulates this expression and makes the T cells sensitive to Fas-mediated apoptosis (26, 33). At the same time, activation by antigens induces FasL expression on cytotoxic T cells (24, 67), which are now able to kill Fas-expressing activated lymphocytes by a Fas-based mechanism. The Fas and FasL may interact on the same cell (66) (Fig. 3B) or on different cells (67) (Fig. 3A). In addition, FasL may be released from the activated cytotoxic cells and then activate Fas from solution (29, 68) (Fig. 3C). In each case, the cytotoxicity is not directed against oneself or modified self, but against activated self.

In the absence of a properly functioning Fas-FasL system, such as in *lpr* or *gld* mutants, activated lymphocytes accumulate (69), and because these cells are not efficiently eliminated, autoimmune disease is enhanced. However, other mechanisms may also contribute to the elimination of activated lymphocytes, and there is evidence that these other mechanisms may predominate in young mice, whereas the Fas-based mechanism may be essential in older mice (70, 71).

B cells are also thought to die by apoptosis at several steps of their development (72). During development in the bone marrow, the B cells that are strongly reactive to self components are deleted, apparently by a Fas-independent mechanism (71). The surviving B cells then migrate to peripheral lymphoid organs where they can be activated by antigen. As with T cells, Fas may be involved in the deletion of B cells activated by the self or foreign antigens in the periphery. Activation of mature B cells causes the expression of Fas (5) and renders the cells sensitive to Fas-mediated killing by anti-Fas (73). It is possible that the FasL on activated T cells binds to Fas on activated autoaggressive B cells and kills them by apoptosis. This process would be blocked in *lpr* or *gld* mice, and the B cells that escape deletion may be responsible for the production of a large quantity of immunoglobulins, including autoantibodies, in these mutant mice.

### Fas-Based T Cell-Mediated Cytotoxicity

Cytotoxic T lymphocytes (CTLs) are the main effector arm of the immune system responsible for eliminating virus-infected cells. The CTLs can both specifically recognize and lyse their targets. What mechanism or mechanisms are involved in lysis? A well-



**Fig. 4.** A schematic representation of two major pathways of T cell-mediated cytotoxicity. The sensitizing antigen, presented by the MHC at the target cell surface, is recognized by the T cell receptor (TCR) at the effector cell surface. The engaged TCR-CD3 complex then transduces within the effector cell a signal toward the granule exocytosis mechanism, and a signal toward the effector cell nucleus that leads to transcription of, in particular, the FasL gene. Swift expression of the FasL protein then leads to engagement of Fas at the target cell surface, signaling target cell death. Interactions of molecules other than TCR-MHC and the Fas-FasL pairs may further modulate the outcome of the effector-target cell encounter. The question marks indicate some of the steps in these processes for which information is particularly incomplete. TCR engagement leads to increased amounts of FasL mRNA, assumed here to be due to increased transcription, although an increase in message stability is not ruled out.

known perforin-granzyme-based mechanism (74) does not account for all examples of CTL killing, so a search has begun for alternative mechanisms.

A clue was provided by the demonstration that a CTL hybridoma subline (d10S) (35), upon activation with phorbol 12-myristate 13-acetate (PMA) and ionomycin, lysed Fas-positive but not Fas-negative cells (18). Additional evidence for the existence of Fas-based cytotoxicity came from blocking experiments with the soluble form of Fas (Fas-Fc) or anti-Fas under nonlytic conditions. With these reagents, many T cell lines were found to exert Fas-based cytotoxicity (32, 34, 75, 76).

It has long been known that part of T cell-mediated cytotoxicity was  $\text{Ca}^{2+}$ -independent (77), whereas the perforin-granzyme-based mechanism was  $\text{Ca}^{2+}$ -dependent at several levels (78). The cytotoxicity by preactivated d10S cells was  $\text{Ca}^{2+}$ -independent (35). The presence of EGTA- $\text{Mg}^{2+}$  in cytotoxicity assays suppresses the perforin-based mechanism and allows one to study the Fas-based mechanism (18), even in situations where both mechanisms would normally operate simultaneously. In the presence of EGTA- $\text{Mg}^{2+}$ , the antigen-specific cytotoxicity expressed by in vitro-raised and in vivo-raised cytotoxic T cells was shown to be Fas-based (18, 79).

The Fas-based cytotoxicity by CTLs can be divided into two processes. First, on recognition of the target cell by the cytotoxic cell, FasL expression is induced in the cytotoxic cell. Second, engagement of FasL on the cytotoxic cells and Fas on the target cell leads to activation of an intrinsic suicide program in the target cell. Interestingly, transfection of FasL cDNA into fibroblast-like recipient cells is enough to render these cells cytotoxic (20), showing that expression of the FasL may be sufficient to make even a nonlymphoid cell cytotoxic.

Which T cells are able to exert Fas-based cytotoxicity? Among the classical CD4<sup>+</sup> T helper (T<sub>H</sub>) subpopulations (80), T<sub>H1</sub> cells can express the FasL and lyse in a Fas-based manner much more readily than T<sub>H2</sub> cells (32, 75). Neither helper T cell population usually expresses the perforin-based mechanism of lysis. In contrast, CD8<sup>+</sup> cells, which are the professional cytotoxic T cells, usually express both the Fas-based and the perforin-based mechanisms (18, 24, 32, 34).

Thus, two mechanisms of cytotoxicity, perforin-based and Fas-based, have been molecularly defined (Fig. 4). There seems to be little cross-talk between these mechanisms, because cytotoxic cells from perforin knock-out mice (79), as well as cytotoxic cell lines that do not express perforin (81), can lyse by means of the Fas pathway, whereas gld cytotoxic cells and lpr target cells can lyse or be lysed, respectively, by

means of the perforin pathway (18, 67, 79, 82). No other mechanism of cytotoxicity has been found, at least in short-term in vitro cytotoxicity tests (79, 81), suggesting that there are only two main mechanisms of T cell-mediated cytotoxicity, Fas-based and perforin-based mechanisms.

### Fas and FasL in Pathology

As described above, the Fas system is involved in eliminating normal activated lymphocytes as well as probably some virus-infected cells. The loss-of-function mutations of Fas or FasL causes activated lymphocytes to accumulate, and in mice this produces autoimmune disease. An injection of antibodies to Fas kills adult mice within a few hours (83). These results suggest that the Fas system may have a role in human pathology in two different ways.

First, diseases may be caused by a malfunction of the Fas system. The lpr and gld mutations in mice are in this category, and several patients have been described with phenotypes similar to that of lpr mice (84). It is possible that these patients have defects in Fas or FasL. Indeed, patients with altered Fas were recently reported (85). The lpr and gld mice produce large amounts of immunoglobulin, including autoantibodies, and develop an autoimmune disease that resembles human systemic lupus erythematosus (SLE) (52). Cheng *et al.* (86) detected an elevated level of the soluble form of Fas in the serum of some human SLE patients. This soluble form of Fas seemed to be produced by a Fas mRNA that was generated by alternative splicing and that encoded a soluble form of Fas protein lacking a transmembrane domain. Because this form of Fas could induce lymphadenopathy and splenomegaly, Cheng *et al.* (86) suggested that it inhibits Fas-mediated elimination of activated lymphocytes and thereby causes the SLE phenotype. Although this is an interesting possibility, it will be necessary to examine more patients to test this hypothesis.

The lpr phenotype is dependent on background genes in the mouse (54). MRL mice carrying the lpr mutation develop nephritis or arthritis, whereas C3H mice carrying the lpr mutation do not, indicating that genes other than Fas, such as those regulating autoimmunity, are involved in Fas-related pathology (87). It may be noteworthy that nitric oxide (NO) synthase expression and NO production are increased in MRL lpr/lpr mice, and spontaneous glomerulonephritis and arthritis can be reduced by orally administered N<sup>G</sup>-monomethyl-L-arginine which inhibits NO production (88). Active transforming growth factor- $\beta$  (TGF- $\beta$ ) is overproduced in lpr mice, possibly as a homeostatic mechanism for suppressing exaggerated and inappropriate

immunostimulation. This apparently leads to a failure of polymorphonuclear leukocytes to migrate to the site of bacterial infection, diminishing the host defense against bacterial infection and thus increasing the bacterial burden (89). This situation is reminiscent of the increased risk of bacterial infection observed in patients with autoimmune disease.

A second category of Fas-related diseases may be caused by excessive activity of the Fas system. There is growing circumstantial evidence that Fas might be involved in the death of CD4<sup>+</sup> T cells during the course of an HIV infection. Fas is abundantly expressed on T lymphocytes of HIV-infected children (15) and in retrovirus-induced immunodeficiency syndrome in mice (90). In unfractionated human peripheral blood lymphocytes (PBLs), cross-linking of CD4 molecules, either by an antibody or by the HIV envelope protein gp160, up-regulated the expression of Fas on the PBLs, which closely correlated with the occurrence of apoptotic cell death (91). Injection of mice with antibody to CD4 led to rapid Fas-based apoptosis of T cells (92). Human T cell lines transformed with HIV were more sensitive to Fas-mediated apoptosis than the parental cells (15). These data are consistent with the involvement of Fas in the pathology of acquired immunodeficiency syndrome (AIDS), and this potentially important line of research is now being actively pursued in several laboratories.

An injection of monoclonal anti-Fas into adult mice caused rapid hepatic failure and death (83), suggesting that acute fulminant hepatitis in humans may be Fas-mediated. Accumulating data such as the involvement of specific CTLs in fulminant hepatitis (93), the sensitivity of primary hepatocytes to Fas-mediated apoptosis in vitro (94), and the overexpression of Fas in hepatocytes transformed with human hepatitis C virus (HCV) (95) are consistent with this hypothesis. In this model, virus antigens of hepatitis B virus or HCV expressed on hepatocytes would activate CTLs to express FasL, which then would bind to Fas on hepatocytes, inducing them to undergo apoptosis. This process may normally occur to remove virus-infected cells but, if exaggerated, may lead to fulminant hepatitis.

Fas is abundantly expressed not only in the liver but also in the heart and lungs (9). The primary cells from these tissues are sensitive to Fas-mediated apoptosis (96), suggesting that the Fas system may also be involved in CTL-mediated diseases in these tissues. The lymphocytes in lpr mice constitutively express FasL (69), which may be responsible for the graft-versus-host disease (GVHD) observed when lpr bone marrow is transferred to wild-type mice (97). These

results suggest that the Fas system may also be involved in GVHD induced by allogenic bone marrow transplants in humans. Furthermore, a functional soluble form of the FasL was recently identified in the culture medium of activated human T cells (29). If such a molecule is produced in vivo, it may work as a pathological agent to cause systemic tissue injury.

Therapeutic uses of the Fas system might include blocking the exacerbated Fas-based pathological manifestations with either soluble forms of Fas, neutralizing antibodies to Fas or FasL, or inhibitors of FasL induction or Fas-mediated apoptosis. Some groups considered using Fas-based reagents (such as activating anti-Fas or FasL) for human cancer patients (15, 98). To avoid the potential risks of these reagents for normal tissues (83), it would be necessary to find a way to accurately target the reagents to the tumor cells.

## Conclusions

Cell proliferation, differentiation, and survival are often regulated by growth, differentiation, and survival factors, respectively, which are collectively called cytokines. Cytokines bind to their complementary receptors, which transduce the extracellular signal into an intracellular signaling cascade. As described above, characterization of Fas and FasL revealed that, in some cases, cytokines can induce cell death. FasL binds to its receptor and kills the cell within hours by inducing apoptosis. Whereas dozens of factors are known to promote growth, differentiation, or survival, only a few cytokines, including FasL and tumor necrosis factor (TNF), have been found to induce apoptosis. The Fas and TNF systems seem to be mainly restricted to the immune system. It is possible that there are other death-inducing molecules functionally related to FasL that may help remove unwanted cells in nonlymphoid tissues and organs.

Binding of a growth factor to its receptor usually induces dimerization of the receptor (homodimerization or heterodimerization). The dimerized receptor then activates a cascade of intracellular reactions, which eventually induce the gene expression necessary for cell proliferation or differentiation. On the other hand, binding of FasL to its receptor probably induces trimerization of the receptor, which can then transduce the death signal. The fact that Fas-mediated apoptosis can occur without a nucleus indicates that no specific gene induction is required for this death process. The cells seem to have an intrinsic death program in the cytoplasm, which Fas may activate. This death program may be identical to that involved in apoptosis induced in other ways, for example, by the deprivation of survival factors. Some cells are resistant to

Fas-mediated apoptosis, suggesting that they either lack this death program or express molecules that inhibit the signals induced by Fas or the death program itself. Biochemical characterization of this death program and its eventual inhibitor proteins in the cytoplasm may reveal other signaling intermediates.

The identification of FasL as a cell death-inducing molecule suggested that the Fas-FasL system has a pathological role in humans. It is known that TNF works as a cachectin and mediates septic shock. Like TNF, FasL may work as an agent that causes tissue damage. The gain-of-function mutation of the growth factor system causes cellular transformation, whereas the loss-of-function mutation of the Fas system (*lpr* or *gld* mutation) causes lymphadenopathy. In this regard, Fas and FasL may be considered as tumor suppressor genes. It is possible that one or more mutations in oncogenes or tumor suppressor genes in addition to the Fas-FasL mutation causes cellular transformation. Further elucidation of the mechanism of Fas-mediated apoptosis and of its role in physiology or pathology should contribute to a better understanding of not only the life and death of cells but also of the basic mechanism of some human diseases.

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## Apoptosis in the Pathogenesis and Treatment of Disease

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In multicellular organisms, homeostasis is maintained through a balance between cell proliferation and cell death. Although much is known about the control of cell proliferation, less is known about the control of cell death. Physiologic cell death occurs primarily through an evolutionarily conserved form of cell suicide termed apoptosis. The decision of a cell to undergo apoptosis can be influenced by a wide variety of regulatory stimuli. Recent evidence suggests that alterations in cell survival contribute to the pathogenesis of a number of human diseases, including cancer, viral infections, autoimmune diseases, neurodegenerative disorders, and AIDS (acquired immunodeficiency syndrome). Treatments designed to specifically alter the apoptotic threshold may have the potential to change the natural progression of some of these diseases.

The survival of multicellular organisms depends on the function of a diverse set of differentiated cell types. Once development is complete, the viability of the organism depends on the maintenance and renewal of these diverse lineages. Within vertebrates, different cell types vary widely in the mechanisms by which they maintain themselves over the life of the organism. Blood cells, for instance, undergo constant renewal from hematopoietic progenitor cells. In addition, lymphocytes and cells within the reproductive organs undergo cyclical expansions and contractions as they participate in host defense and reproduction, respectively. In contrast, neural cells have at best a limited capacity for self-renewal, and most neurons survive for the life of the organism.

Within each lineage, the control of cell number is determined by a balance between cell proliferation and cell death (Fig. 1). Cell proliferation is a highly regulated process with numerous checks and balances. For example, growth factors and proto-oncogenes are positive regulators of cell cycle progression (1). In contrast, tumor suppressor genes act to oppose uncontrolled cell

proliferation (1, 2). Tumor suppressors can prevent cell cycle progression by inhibiting the activity of proto-oncogenes. In the last 15 years there has been a rapid increase in our understanding of the mechanisms that control cell proliferation.

Biologists are now beginning to appreciate that the regulation of cell death is just as complex as the regulation of cell proliferation (3). The differentiated cells of multicellular organisms all appear to share the ability to carry out their own death through activation of an internally encoded suicide program (4). When activated, this suicide program initiates a characteristic form of cell death called apoptosis (5, 6). Apoptosis can be triggered by a variety of extrinsic and intrinsic signals (7) (Fig. 2). This type of regulation allows for the elimination of cells that have been produced in excess, that have developed improperly, or that have sustained genetic damage. Although diverse signals can induce apoptosis in a wide variety of cell types, a number of evolutionarily conserved genes regulate a final common cell death pathway that is conserved from worms to humans (8) (Fig. 3).

Apoptotic cell death can be distinguished from necrotic cell death (4–6). Necrotic cell death is a pathologic form of cell death resulting from acute cellular injury, which is typified by rapid cell swelling and lysis. In contrast, apoptotic cell death is

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